JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

Volume 23

Number 4

October 1959

The Relation of Medical School Teaching Methods to Student Performance on a Test of Cancer Knowledge [1, 2]

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SUMMARY

Twenty-eight 4-year medical schools completed a questionnaire indicating the number of hours their students spent in classes devoted entirely to cancer. The schools indicated the number of hours students spent: (1) listening to lectures, (2) participating in discussions, (3) doing laboratory work with tumors, (4) observing others work with patients, and (5) working with patients. For each of these methods, Cancer Knowledge Examination scores of students in schools reporting many or some hours of instruction were compared with scores of students in schools reporting few hours or no such instruction. An attempt was made to isolate the effects of each method from those of the other methods. The hours of instruction in the first 2 years were too scattered to permit interpretation of the differences in scores. Among juniors, higher scores were obtained by those having laboratory work and work with patients. The average for those having more than 7 hours of lectures was slightly higher than for those having less than 7 hours. Those who spent more than 10 hours watching others work with patients had lower scores than the students who spent less than 10 hours. For seniors, discussions, laboratory work, and working with patients were associated with higher scores, but listening and observing were not. The results of the senior students were the most reliable. It is suggested that these special courses helped students organize and integrate their knowledge about cancer. The approaches not well adapted to this purpose appeared inefficient.—J. Nat. Cancer Inst. 23: 605–616, 1959.

TRADITIONALLY, CANCER, like most diseases, has been taught "horizontally" (in regular courses) in several departments of the medical school, with only the student coordinating and integrating the information presented to him. A 1946 report of a committee of the National Advisory Cancer Council suggested that the cancer training received by most medical students could be improved, and that a revision of this traditional

[1] Received for publication May 20, 1955; revised April 29, 1959.

[2] This study was supported by grants CS-1020 (C5) and CT-493 (C6) from the National Cancer Institute, National Institutes of Health, Public Health Service.

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approach in the direction of better coordination and integration of cancer instruction would be advisable (*1*). As a result of this report, the Committee on Cancer in the Medical School Curriculum was formed.

The report of this Committee (*2*) recommended that: (*a*) Each school appoint a Cancer Coordinator from among its professors; (*b*) each school review its method of teaching cancer; (*c*) an integrated course in cancer be given in each school, preferably in the senior year; (*d*) cancer research in the schools be stimulated, and (*e*) the United States Public Health Service supply financial support for this program.

As a result, a number of schools inaugurated special courses, such as cancer lectures, tumor clinics, and conferences, in an attempt to coordinate and integrate the teaching of cancer as recommended by the Committee. Although the report of the Committee concerned itself primarily with instruction in the senior year, changes were also made by many schools in the earlier portions of their program. Therefore, data for this study were collected for all 4 years.

The 150-item "Examination for Students of Medicine in the Subject Matter of Cancer" (*3–6*), administered annually to students in medical schools throughout the country (over 17,000 students in 59 schools in 1953), provided data for evaluating these changes.

A preliminary report on the effectiveness of some of the cancer teaching methods at the junior-class level, with the examination scores as the criterion of knowledge, was made by Bierman *et al.* (*6*) in 1952. The present study is an extension of that investigation. This report will present the data, class by class, with emphasis on the senior-class results, since it is the knowledge of the graduating senior that necessarily concerns us most.

PROCEDURE

A questionnaire was sent to all medical schools participating in the testing program. The schools were requested to report the number of class hours spent on the subject matter of cancer during the 1952–53 academic year. They were asked to indicate separately, for both their regular courses and their special cancer courses, the number of hours spent by their students:

(1) Listening to others talk about cancer, *e.g.*, in lectures.

(2) Talking with others about cancer, *e.g.*, in seminars or discussion groups.

(3) Working with tumors but not patients, *e.g.*, in pathology laboratories.

(4) Watching others work with patients, *e.g.*, in clinics and wards run exclusively by doctors.

(5) Working with patients, *e.g.*, in clinics and wards where the students participated directly.

Twenty-eight usable replies were received. These 28 schools were all 4-year institutions and reasonably representative of the 59 schools tested [*cf.* Bierman *et al. (6)*] with respect to size, location, and mean scores on

the test in each class. Data concerning type of students, faculty, physical facilities, and clinical material available for teaching were not obtained.

A preliminary analysis of the total hours spent in regular and special courses for freshman, sophomore, junior, and senior classes indicated that the special courses were more closely related to test scores than the regular courses were. Similar variance analyses for each class led to the same conclusion. Therefore, further analysis was restricted to scores in relation to hours in special classes.

The median number of special course hours spent in each of the 5 procedures was determined for each class (table 1), and every school was classified as above or below the median in relation to each of the 5 methods. Thus every school was classified 5 times in each class. The schools were then grouped according to these classifications. Since lecturing was the only method used at the freshman level, these data are not shown in table 1.

TABLE 1.—Hours of instruction in special cancer courses according to teaching methods used

Method of instruction	Number of schools with no special course hours	Number of schools with some special course hours	Range of hours below median	Median hours	Range of hours above median
		*Sophomores**			
1	16	12	0	0	3–32
2	25	3	0	0	1–6
3	22	6	0	0	6–32
4	26	2	0	0	3–36
5	28	0	—	—	—
Total, all methods	13	15	0	3	4–64
		Juniors†			
1	9	18	0–4	7	8–36
2	16	11	0	0	2–176
3	22	5	0	0	4–26
4	15	12	0	0	5–33
5	18	9	0	0	2–33
Total, all methods	1	26	0–17	20	22–242
		*Seniors**			
1	12	16	0	3	5–42
2	14	14	0	1	2–32
3	23	5	0	0	2–24
4	10	18	0–6	10	12–97
5	10	18	0–6	8	10–40
Total, all methods	2	26	0–30	30. 5	31–137

*Twenty-eight schools.
†Twenty-seven schools.

Grouping the schools according to methods 1, 2, 3, and 4 for sophomores, and 1, 2, 3, 4, and 5 for juniors and seniors gave results ranging from schools below the median, on every method, through a variety of combinations up to those above the median on every method. The examination scores of the students receiving these types of instruction were then compared by means of analysis of variance. The specific statistical method used was that for disproportionate subgroup numbers

as outlined by Snedecor (*7*, pp. 248–289). The many missing possible combinations meant that the methods had to be compared two at a time in each class.

RESULTS

Results for each class will be discussed separately. For convenience it will be assumed in presenting the results that the teaching methods were causal factors in the differences obtained (*cf.* Discussion). Mean test scores for each combination of methods can be found in table 2. Few combinations can be found at all levels and many possible combinations were not used at all. Results of the analyses of variance are shown in table 3.

TABLE 2.—Mean test score by class for each combination of teaching methods used*

Method† 1	2	3	4	5	Sophomore: Number of schools	Sophomore: Mean	Sophomore: Rank	Junior: Number of schools	Junior: Mean	Junior: Rank	Senior: Number of schools	Senior: Mean	Senior: Rank
B	B	B	B	B	13	71.3	8	4	92.1	12	2	101.8	12
B	B	B	B	A	0	—	—	1	107.6	1	0	—	—
B	B	B	A	B	1	75.4	3	3	92.9	10	4	97.3	14
B	B	A	B	B	1	73.8	5	2	100.5	3	0	—	—
B	A	B	B	B	0	—	—	1	86.0	14	3	106.4	4
A	B	B	B	B	6	73.3	6	4	94.8	9	0	—	—
B	B	B	A	A	0	—	—	1	98.1	5	3	104.8	7
A	B	B	B	A	0	—	—	0	—	—	3	105.1	6
B	A	B	A	B	0	—	—	0	—	—	2	108.6	3
A	B	B	A	B	0	—	—	0	—	—	1	99.0	13
B	A	A	B	B	1	78.2	1	0	—	—	0	—	—
A	B	A	B	B	3	74.1	4	0	—	—	0	—	—
A	A	B	B	B	2	72.3	7	2	92.2	11	1	102.7	11
B	A	B	A	A	0	—	—	1	91.6	13	0	—	—
A	B	B	A	A	0	—	—	1	96.6	8	1	104.4	8
A	A	B	B	A	0	—	—	0	—	—	2	103.3	9
A	B	A	A	B	1	77.2	2	0	—	—	0	—	—
A	A	B	A	B	0	—	—	1	97.0	7	0	—	—
A	A	A	B	B	0	—	—	1	105.1	2	1	103.0	10
A	A	B	A	A	0	—	—	3	97.7	6	1	106.0	5
A	A	A	B	A	0	—	—	—	—	—	2	108.9	1
A	A	A	A	A	0	—	—	2	98.8	4	2	108.8	2
Total					28	72.1		27	95.6		28	104.1	

*Combinations not shown, such as $A_1A_2A_3A_4B_5$, were not used by any school for any class.
†A = more than the median number of hours (*cf.* table 1).
B = less than the median number of hours.

Freshmen

Since freshman special courses in cancer were limited to lecture courses, only the regular *versus* special comparison, which favored the special courses, could be made. The regular hours also were almost exclusively in lecture courses. The differences due to the use or nonuse of special cancer lectures at this level are of dubious educational significance, since the statistically significant difference favoring special classes was less than

TABLE 3.—Results of the analyses of variance

Teaching methods compared			Significance level (P)*					
			For Method X alone (effect of Y held constant)			For interaction of X and Y		
Method X	*versus*	Method Y	Soph.	Jr.	Sr.	Soph.	Jr.	Sr.
Lecture (#1)		Discussion (#2)	01†	01	NS	01	01	01
Lecture (#1)		Laboratory (#3)	NS	01	—‡	NS	05	—
Lecture (#1)		Watching (#4)	NS	01	NS	NS	01	NS
Lecture (#1)		Working (#5)	—	NS	NS	—	01	05
Discussion (#2)		Lecture (#1)	01	01†	01	01	01	01
Discussion (#2)		Laboratory (#3)	05	05†	—	NS	NS	—
Discussion (#2)		Watching (#4)	—	NS	01	—	01	01
Discussion (#2)		Working (#5)	—	01†	01	—	01	01
Laboratory (#3)		Lecture (#1)	01	01	—	NS	05	—
Laboratory (#3)		Discussion (#2)	01	01	—	NS	NS	—
Laboratory (#3)		Watching (#4)	01	01	01	NS	01	NS
Laboratory (#3)		Working (#5)	—	01	01	—	01	NS
Watching (#4)		Lecture (#1)	01	NS	NS	NS	01	NS
Watching (#4)		Discussion (#2)	—	01	NS	—	01	01
Watching (#4)		Laboratory (#3)	01	NS	05†	NS	01	NS
Watching (#4)		Working (#5)	—	01†	05†	—	01	01
Working (#5)		Lecture (#1)	—	01	01	—	01	05
Working (#5)		Discussion (#2)	—	01	01	—	01	01
Working (#5)		Laboratory (#3)	—	NS	01	—	01	NS
Working (#5)		Watching (#4)	—	01	01	—	01	01

*As determined from Tables of F provided by Snedecor (7). Any P greater than 0.05 was considered insignificant (NS).

†Schools using Method X had lower scores than those not using the method.

‡Incomplete data.

one point in mean score. The large number of students considered (2,114) and the typically small variance in freshman scores are reasons for the significance of this difference.

Sophomores

Special classes in cancer were also relatively rare at the sophomore level, which permitted single schools to influence strongly the results of the analyses. In all comparisons between two methods of instruction, more than half the schools fell into the group using neither method. In fact, 13 of the 28 schools had no special cancer courses for their sophomores. The average test scores for these 13 schools were in all instances lower than those for the other 15 schools (*see* table 2), which suggests again, as was previously indicated, that some special instruction in cancer is better than none.

The results of the analyses of variance for sophomores shown in table 2 should be interpreted with great caution. It seems clear that the test

scores at schools having special laboratory work in cancer were higher than those that did not. Less clear differences favor observation of others' work with patients. The other significant differences were in each instance attributable to one single school or to the differences in hours in the laboratory (Method 3). Since individual schools differ in many respects, only the results for Method 3 and possibly those for Method 4 should be taken seriously.

Juniors

The data for the junior-class students present a somewhat more complex picture than for the sophomores. Taking the methods in order, Method 1 (listening) proved to be of some value. Although Method 1 apparently had no significant effect when the influence of working with patients is eliminated, examination of the means reveals that this was the result of the hidden negative effects of Method 4 (observation) on Method 5. It does not appear that Methods 1 and 5 supplement each other.

Method 2, as indicated in table 3, had a significant effect when the effects of Methods 1, 3, and 5 were controlled. The direction of this effect was negative. The explanation for this apparent negative effect of Method 2 can be inferred from the fact that, when Method 2 was not employed, Methods 3 and 5 were utilized. Therefore, we may conclude that by itself, Method 2 contributed relatively little.

It is interesting to note in table 3 that laboratory work with tumors, although not commonly used at the junior level, was effective here just as it was with the sophomores.

Method 4 had a significant effect when contrasted with either Method 2 or Method 5. As already suggested, all the effects apparent in the comparisons of Methods 2 and 4 were the hidden ones of Methods 3 and 5. The effects of Method 4, when contrasted with Method 5, were negative. This interference of Method 4 with Method 5 cannot be explained by the hidden effect of any other method of instruction. Method 4, when considered in conjunction with Method 3, offered no independent contribution to cancer learning and, in fact, appeared to interfere with the effectiveness of Method 3. One must conclude therefore, that at the junior level, watching others work was not a profitable procedure.

Method 5 (working with patients) contributed significantly to cancer knowledge at the junior level. The apparent lack of a significant effect for this method, when Method 3 was held constant, appears to be due to the interfering effect of Method 4 on Method 5. Furthermore, the significant interactions of Method 5 with the others was in each instance the result of interference from Method 4. In short, working with cancer patients was an effective method of instruction.

Thus, for the juniors, one may say that Method 1 (listening to lectures) was fairly useful, and Methods 3 (laboratory work with tumors) and 5 (working with patients) were very effective. Methods 2 (seminars and discussions) and 4 (watching others work) were of no use by themselves, and Method 4 actually interfered with Method 5.

Seniors

At the senior level, listening to lectures appeared to have little value (table 3). None of its independent effects were significant, though it did facilitate the effectiveness of Method 5 and interfered slightly with the efficiency of Method 2.

Seminars and discussions (Method 2) for the seniors, unlike the preceding classes, were clearly of value with regard to cancer-test performance. In fact, the difference in test scores favoring Method 2 at the senior level was the largest found in any of the comparisons reported in this paper. Not only did this type of instruction have unique value, but it also was facilitated by the use of Methods 4 (watching others at work) and 5 (working with patients). We regret that insufficient data prevented a statistical comparison between Methods 2 and 3, but the available data do not contradict the preceding statements.

Method 3 (laboratory work with tumors) was effective in those comparisons in which Method 4 or 5 was held constant. As was true for the sophomores, Method 3 had no significant interaction with the other procedures and may be used to advantage regardless of other teaching methods.

The apparently significant effects of Method 4, when Method 3 was held constant, came about because of the influence of Methods 2 and 5. Furthermore, the significant results for Method 4, when Method 5 was held constant, were also apparently the result of Method 2. None of the other comparisons for Method 4 are significant, and it may be concluded that watching others work with cancer patients had little value at the senior level.

Working directly with cancer patients (Method 5) was effective when each of the other methods was held constant. Furthermore, its interactions with the other methods increased the effectiveness of those methods of instruction.

To summarize for the seniors, Methods 2 (seminars and discussions), 5 (work with patients), and 3 (laboratory work with tumors) were effective techniques of instruction, in that order of importance. Neither of the two passive methods, listening (Method 1) and watching (Method 4), contributed anything significant to cancer learning at this level.

The results for the three upper classes may be summarized, by method, as follows:

1. Listening to lectures: for sophomores—apparently no influence; for juniors—some help; for seniors—no influence except possibly some help in combination with working with patients.

2. Seminars and discussion: for sophomores—unknown; for juniors—little influence; for seniors—a strong positive effect, especially along with working with patients.

3. Laboratory work with tumors: for sophomores—the most effective method; for juniors—quite effective; for seniors—fairly effective.

4. Watching: for sophomores—possibly effective; for juniors—no effect except interference with Method 5; for seniors—no effect.

5. Working with patients: for sophomores—no data; for juniors—very effective; for seniors—effective, especially in combination with Method 2.

DISCUSSION

In the evaluation of results of comparisons among special cancer teaching methods, a number of limitations should be kept in mind:

1. The criterion measure was a paper-and-pencil test whose validity as a test of knowledge is well established (*cf. 6*), but whose value as a predictor of on-the-job performance is unknown. In short, the value of these data hinges upon the importance of sheer knowledge about cancer.

2. Hours of instruction in regular courses were not considered. It was found that the amount of instruction in special courses was more closely related to test scores than the amount in regular courses, but this does not imply that cancer instruction should be largely or solely given in such special courses. Most of a student's knowledge about cancer almost certainly is obtained in the regular courses. The special courses have, for the most part, been superimposed on the regular curriculum and therefore allow no conclusion as to what the effect of replacing regular cancer instruction with special courses would be. Undesirable effects might well follow such a procedure, since the opportunity to see the disease in perspective and in its relation to other diseases would be lost. This could hamper a student's attempt to organize his knowledge about cancer.

3. Class hours in preceding years were not considered for any classes. It was found that the total number of hours of instruction in cancer for all four classes was related to the scores of seniors (who may or may not have received all this instruction). Both amount and quality of prior instruction should influence the effectiveness of any method of instruction. It is possible that the apparent effectiveness of a method was due to the pattern of previous instruction. When more than one school was responsible for the obtained differences, the pattern of previous instruction appears quite unlikely to influence the effectiveness of a method, since no two schools had the same pattern in even the junior and senior years alone. It is more probable that such background differences reduced the differences in test scores between schools.

4. The differences among schools within groups were generally large, and unquestionably certain teaching methods would be more effective or appropriate for some schools than for others. Furthermore, the best combination of methods might be one of those not used by any school in this sample, since each school used only one of the possible combinations of methods.

In spite of these limitations, the data may be of some value if reasonable explanations for the various statistically significant differences can be found.

First, it is possible that the apparent superiority of certain teaching

methods merely reflects current practices among the generally superior schools, *i.e.*, those with the best facilities, the most select student body, etc. If this is true, the best schools would probably (*cf.* limitation in #3) rank high each year. The rank-order correlations (rho) between the standing of the schools for each class do not support this hypothesis strongly. The freshman-senior rho was 0.56, which indicated a significant but far from perfect relationship. Thus, for example, the schools whose freshmen means ranked 1, 2, 3, 4, and 5 had senior means ranking 6, 1, 18, 7, and 27, respectively. The other correlations ranged from 0.23 (freshman-sophomore) to 0.66 (junior-senior). Therefore the over-all quality of the schools had some influence on test scores, but probably do not account for the differences between schools using different methods.

Second, if one can show that the apparent results are consistent, logical, and in agreement with other data on methods of teaching and learning, it can be assumed that the particular procedures were influential. With the help of previous studies this appears to be possible.

In the Bierman *et al.* (*6*) study of junior scores, attendance at tumor boards, work with patients in clinics, and formal lectures in the sophomore year were the variables considered. No clear general pattern could be found, but the importance of patterns was strongly indicated. Also it was found that, in each of the three upper classes, fewer of the highest scoring schools than of the lowest scoring schools had formal lectures and more required an above average number of hours of clinical work.

Towner (*8*) found that medical students' knowledge of cancer progressed gradually from specific, academic, factual knowledge to organized clinical knowledge. Thus, differences in test scores were largely due to the former during the first two years, whereas differences among seniors were more likely to be differences in the degree of generality and organization of this knowledge.

The present data are consistent with these studies. Neither seminars, which emerged by the senior year as most characteristic of high-scoring schools, nor working with patients, which appeared to contribute in both the junior and senior years, can be expected to make as much of a contribution to a student's store of specific information as lectures. Most studies comparing discussions with lectures at the college level confirm this statement (*9*). Thus it seems probable that the function of special courses in Methods 2 and 5 is to organize and integrate information already acquired.

Laboratory research has shown that extensive guidance in the early stages of learning is constructive, but that, as the student progresses, it may come to interfere with learning (*10*). Thus one might expect that demonstrations classified under Method 4 would be helpful primarily as an initial step in most kinds of clinical training. On the other hand, the opportunity to try one's own ideas, to make, discover, and correct one's own mistakes should be more helpful in the later stages of learning. The results for Methods 4 and 5 are consistent with these generalizations from laboratory studies of learning.

The superiority of laboratory work with tumors (Method 3) can be accounted for in two different ways, both of which may be true: (*a*) One may say that the material learned in the pathology laboratory is highly detailed and specific. Such material would then need frequent review if it is to be recalled sufficiently well to be useful (*10*). (*b*) One may say that this material provides the theoretical foundation for organizing and understanding the entire field. To the extent this latter suggestion is accurate it would appear that a good deal more work of this nature is needed in most schools. Method 3 did not interact with the other methods (the significant interactions with Methods 4 and 5 at the junior level are artifacts of the statistical procedure) which suggests that the first explanation is the more likely one. Otherwise one would expect such studies to enhance markedly the value of the other approaches, *i.e.*, to interact with them.

Also consistent with this proposed pattern of cancer learning are the results with respect to lectures for the juniors and seniors. As one would expect, special lectures appeared to be of some help in the junior year, but of little or no value to the seniors. However the results for Method 1 (lectures) at the sophomore level do not fit this pattern. The sophomores did not, as the hypothesis would lead one to expect, appear to profit extensively from attending lectures on cancer. Thus, although the data fit the pattern for the other 4 methods at all 3 levels and for lectures at 2 of the 3 levels, it would not be unreasonable to reject the hypothesis that the differences in test scores were the result of the differences in teaching methods among the schools. However, in view of the lack of precision and control in this study, which makes one inconsistency more likely than not, it is also not unreasonable to attribute these results to the interference of extraneous factors. For example, the lectures may have required a background the students did not have; they may have dealt with matters the students did not believe were relevant to their main task (*11*); or it may be that the students were surfeited with lectures.

Also relevant is a recent report (*12*) in which 8 schools with regular courses only were compared with 17 schools with both regular ("horizontal") and special ("vertical") courses. The comparison of sophomore to senior gains in test score favored the schools with regular courses only.[5] However, 7 of these 17 schools with both approaches did not offer any special courses in the senior year and 3 more offered lectures only; such arrangements also produced lower scores among the schools in the present study. Two of the 17 schools gave seminars to their seniors, presumably a profitable approach. The courses given in the 5 remaining schools were described as conferences and as cancer or oncology courses. Whether these involved listening, discussing, observing, or working with patients is not clear. Laboratory work was apparently

[5] The data reported in this paper showing that differences between schools with both arrangements were more closely related to hours of special instruction than to hours of regular instruction do not necessarily contradict this conclusion. It may be noted that the mean number of hours of special instruction for the 17 schools was 31, in contrast to the median of 62 hours for the 28 schools considered in the present report.

not included. Thus only a minority of the instruction was of the variety which the foregoing interpretation describes as favorable. It must therefore be emphasized that special courses will lead to higher test scores only if they are taught in a manner appropriate to their major purpose: the organization and integration of information and principles by senior students. It is also possible to infer that, while special junior courses may influence junior scores, they have little effect on senior scores.

A second possible interpretation of the data is that the teaching methods used by a school strongly influenced the performances of its students on the Cancer Knowledge Examination. In the early stages of learning, the superior methods were those adapted to maximum guidance of the student's learning and to the rapid acquisition of information, *i.e.*, observations and, theoretically, lectures. Gradually there is a change as the student's major task becomes one of organizing and integrating his information and acquiring the ability to draw inferences from it. A consequence of this change is the increased usefulness of methods permitting the student greater freedom to participate, to make and correct errors, and to interpret facts, *i.e.*, by seminars and working with patients. The consistent superiority of laboratory work with tumors can be fitted to this pattern in two different ways, as already noted.

Finally, the differences between schools with various patterns of instruction may be due not to the inferiority or superiority of the methods in and of themselves but to the attitudes of the faculty, which the adoption of these approaches implies. Particularly at the senior level, it was the schools with methods which entail work with small groups and individuals that had the highest scores. A faculty sufficiently interested in teaching and in their students to put in the time and effort required might well be expected to produce superior students. This explanation probably supplements the second.

In evaluating these explanations it should be emphasized that the results for seniors should be given the greatest weight and attention, primarily because the students' knowledge and skill at graduation is the school's greatest concern. In addition, because the distribution of schools among the four methods considered (ignoring Method 3) was more even at the senior level, it did not happen that any one school determined the results as did occur within several of the sophomore comparisons and a few of the junior comparisons. Furthermore, the results for the seniors are more readily interpretable, since the hidden effects of methods not under consideration had less influence on the statistics at this level. Thus, the results for the seniors are of greater educational significance than the ones found for the other classes.

Therefore, the available data suggest that the proposals made by the Committee on Cancer in the Medical School Curriculum for special integrated cancer courses for seniors were profitable when adopted, particularly if the teaching methods required the active participation of the students. It seems likely that many schools without such courses would find it advantageous to adopt similar procedures. To a lesser extent,

the same statements may be made for the juniors, with the proviso that greater efforts be made to correlate and coordinate these special courses with each other. More adequate data are necessary before any recommendations can reasonably be made for sophomore classes. Too little is known about cancer learning in the freshman year to make any broad generalizations.

REFERENCES

(*1*) National Advisory Cancer Council: Cancer facilities and services; a report from the National Advisory Cancer Council. J. Nat. Cancer Inst. 6: 239–302, 1946.

(*2*) National Advisory Cancer Council: Cancer in the medical school curriculum. J. Nat. Cancer Inst. 8: 1–6, 1947.

(*3*) BIERMAN, H. R., and MCCLELLAND, J. N.: A study of methods for the improvement of cancer learning in the medical school; first annual report. J. Assoc. Am. M. Coll. 24: 351–362, 1949.

(*4*) ———: An achievement examination in the subject matter of cancer for medical school student. Pub. Health Rep. 66: 769–778, 1951.

(*5*) BIERMAN, H. R., MCCLELLAND, J. N., and GALLOWAY, D. W.: Assessment of student knowledge on the subject matter of cancer. J. M. Educ. 27: 272–277, 1952.

(*6*) BIERMAN, H. R., TOWNER, L. W., GALLOWAY, D. W., and MCCLELLAND, J. N.: Cancer Learning in the Medical School. University of California Press, Berkeley, 1952, pp. 5–87.

(*7*) SNEDECOR, G. W.: Statistical Methods Applied to Experiments in Agriculture and Biology, 4th ed. Ames, Iowa, The Collegiate Press, Inc., 1946, pp. 16–485.

(*8*) TOWNER, L. W.: A factorial investigation of medical students' knowledge of cancer at different levels of training. Ed. Psych. Meas. 17: 115–130, 1957.

(*9*) RUJA, H.: Experimenting with discussion in college teaching: A survey of recent research. Ed. Admin. and Sup. 39: 321–342, 1953.

(*10*) MCGEOCH, J. A., and IRION, A.: The Psychology of Human Learning, 2d ed. New York, Longmans, Green & Co., 1952, pp. 24–596.

(*11*) BECKER, H., and GEER, H.: Student Culture in Medical School. Harvard Ed. Rev. 28: 70–80, 1958.

(*12*) LORET, P. G., RANKIN, R. J., and WILCOX, N. E.: The cancer achievement testing program. II. A comparison of the effectiveness of vertical and horizontal cancer instruction. J. M. Educ. 34: 523–528, 1959.

An Appraisal of Left and Right Breast Cancer [1]

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SUMMARY

The literature on the occurrence of left and of right breast cancer in the United States and in foreign countries is reviewed. In addition, several new series of cases from cancer registries and hospitals are reported. An analysis of the data shows that breast cancer in women occurs more often in the left breast than in the right breast. This difference is greater in foreign countries than in the United States. It is also shown that there is a smaller left/right ratio for breast cancer in women over the age of 70; that there are regional differences in the United States, by color; and that single women appear to have lower left/right ratios for breast cancer than women who are ever married. In American males, the occurrence of left breast cancer over that of right breast cancer shows a greater difference than in females. Trauma, poor milk secretion, and other factors are discussed in relation to the observed differences by side of breast.—J. Nat. Cancer Inst. 23: 617–631, 1959.

IT HAS been generally accepted that left breast cancer occurs more frequently than right breast cancer. Busk and Clemmesen (*1*) studied the incidence of left and of right breast cancer in the records of the Danish Cancer Registry for a 5-year period, compared their results with two other series of cases, and concluded that cancer of the left breast was about 11 percent more common than that of the right breast. Smithers (*2*) compared a series of breast-cancer cases in the Royal Cancer Hospital in England, for a 12-year period, with three other series and came to the same conclusion. In this country, Dorn and Cutler (*3*) examined the results of cases obtained in their 10-city morbidity study, 1947–48, and found that left breast cancer occurred 8 percent more frequently than right breast cancer.

[1] Received for publication March 6, 1959.

[2] The authors wish to thank Dr. Arthur Purdy Stout for permission to abstract records at the Francis Delafield Hospital, and Dr. G. J. Tarleton for access to the Meharry Medical College records. Dr. Vincent Handy, Dr. Beulah Hathaway, Dr. George Adie, and Mrs. Marion Wogan permitted us to use the records of the New York State Cancer Registry, the Alabama Pathologists' Association Cancer Registry, the Westchester County Cancer Registry, and the Charity Hospital Cancer Registry, respectively. We also wish to thank Miss Eleanor MacDonald of the M. D. Anderson Hospital, in Houston, and Mr. Sidney Cutler of the National Cancer Institute, for specially prepared tabulations.

This study was undertaken because we felt that this difference in "sidedness" in breast cancer had not been sufficiently explored. If the difference in the incidence pattern of left over that of right breast cancer is a real one, a more intensive examination of the phenomenon could conceivably uncover information of etiologic significance, or possibly give leads to further research. A difference of 11 percent in the occurrence of cancer in the two breasts, apparently similar in physiology and anatomy, if true, would imply that some contributing factor or factors exist. This percentage difference, although small, is not inconsiderable in terms of total number of cases. Further investigation of differences in subgroups, such as age, sex, color, marital status, etc., can potentially uncover larger differences, which can then be further investigated. There is a voluminous literature on the relationship of breast cancer to these attributes, although very little exists relating them to side of breast.

We first decided to examine the material published on the subject to see whether the left/right difference held in several series of cases. In addition, we included unpublished breast-cancer cases from the records of a number of cancer registries, and were able to obtain original records from the Francis Delafield Hospital in New York City and the Meharry Medical College in Nashville.

The data are presented in tables 1 and 2. The occurrence of left *versus* right breast cancer in foreign countries showed a pattern of consistency. The left/right ratios for 9 series of cases ranged from 1.09 in Sweden to 1.18 in Japan. For the total of 18,173 cases in 7 countries, the over-all ratio was 1.13.

The American experience, presented separately in table 2, shows an over-all left/right ratio of 1.04 for 15,931 cases. In 18 series of cases, 8 series showed more right breast-cancer cases than left. The left/right breast-cancer ratios varied from 0.74 to 1.36.

This range of ratios is much greater than that shown for the foreign countries, but, of course, the total number of cases in many of the series is small and therefore subject to greater random fluctuations. If one were to consider only the 7 series of cases in the American data that have as many cases as the smallest in the foreign country data, the range of left/right ratios is from 0.89 to 1.08 and the over-all ratio is 1.03.

Left breast cancer in the total American experience, in table 2, is significantly greater than an equal distribution by side of breast ($P = 0.02$). The American ratio of 1.04 is significantly different from the total foreign left/right ratio of 1.13 ($P = 0.001$).

On the surface, there seems to be no reason why the American data should differ from that of other countries. Certainly the series we have collected in the United States differ with respect to region in which obtained, by proportion of white and colored, and probably have much different distributions with respect to age, marital status, and other factors. There is no reason, however, to suspect that they are more heterogeneous in these respects than groups of cases collected in England, Italy, Colombia, and Japan. Yet not only is there an over-all difference

TABLE 1.—Ratios of occurrence of left and of right breast cancer among females in foreign countries

	Total cases	Left breast	Right breast	Bi-lateral	Not stated	Left/right ratio	Source	Country	Description of cases
1.	1042	537	490	15	—	1.09	Nohrman (*4*)	Sweden	All cases seen in Radiumhemmet, Stockholm, 1936–41
2.	2129	1114	1005	6	4	1.11	Hartnett (*5*)	England	All hospitals in administrative county of London, 1938–39
3.	4139	2117	1908	97	17	1.11	Busk & Clemmesen (*1*)	Denmark	All hospital cases reported to Danish Cancer Registry, 1942–46
4.	486	256	230	—	—	1.11	Valderama *et al.* (*6*)	Colombia	Cases seen at the Instituto Nacional de Cancerologia, 1946–52
5.	425	218	195	5	7	1.12	Ballarati (*7*)	Italy	Cases seen at the Ospedale di Circolo e Centro Tumori di Busto Arsizio, Milan, 1921–50. Total includes 7 males.
6.	1045	522	464	22	37	1.13	Fellenberg (*8*)	Switzerland	Cases reported to the Swiss Cancer Census, 1933–35
7.	1762	910	799	52	1	1.14	Smithers *et al.* (*2*)	England	Cases seen at the Royal Cancer Hospital, London, 1937–48
8.	6530	2995	2623	643	269	1.14	Registrar-General's Report (*9*)	England & Wales	All deaths from cancer of the breast in England and Wales, 1931–34
9.	615	314	265	24	12	1.18	Segi *et al.* (*10*)	Japan	All cases seen in Medical School Hospitals in Japan, 1948–52
TOTAL	18,173	8983	7979	864	347	1.13			

TABLE 2.—Ratios of occurrence of left and of right breast cancer among females in the United States

	Total cases	Left breast	Right breast	Bilateral	Not stated	Left/right ratio	Source	Location	Description of cases
1.	134	57	77	—	—	0. 74	Deaton *et al.* (*11*)	North Carolina	Patients operated on in North Carolina Baptist Hospital, 1941–47. One male included.
2.	121*	56	71	—	—	0. 79	Antonellis (*12*)	Massachusetts	Consecutive cases treated in Myers Clinic, 1935–53. Includes 2 cases of male breast cancer.
3.	833	372	416	45	—	0. 89	Kaplan & Rosh (*13*)	New York, N.Y.	Cases accepted for treatment in 20-year period (1925–44). Includes 83 Negroes, 2 Chinese; 19 males.
4.	402	188	202	12	—	0. 93	Siris & Dobson (*14*)	San Francisco, Calif.	Cases seen in the clinics of Stanford University Hospital from 1926–45. Includes 7 cases of male breast cancer.
5.	100†	51	54	—	—	0. 94	Kennedy & Jelks (*15*)	Jacksonville, Fla.	Cases seen in Duval County Hospital from 1926–47. Includes 76 Negroes, 24 whites; 3 cases of male breast cancer.
6.	544	261	277	6	—	0. 94	Anderson *et al.* (*16*)	Minnesota	Cases examined at the Tumor Clinic of University of Minnesota Hospital, with microscopic diagnosis or clinical, inoperable breast cancer. White women only included.
7.	1205	476	508	7	214	0. 94	—	New Orleans, La.	Cases in the Cancer Registry, Charity Hospital, New Orleans, 1948–57. Includes 747 Negroes and 458 whites.
8.	1117	481	500	—	136	0. 96	—	Westchester Co., N.Y.	Cases reported to the Central Cancer Registry in Westchester County by 7 reporting hospitals, 1948–58. Includes 56 colored females.
9.	191	95	92	1	3	1. 03	—	Nashville, Tenn.	Cases seen at the Meharry Medical College Tumor Clinic, Hubbard Hospital, Nashville, Tennessee, 1946–58. All colored females.
10.	1056	544	509	—	3	1. 07	Shimkin *et al.* (*17*)	California	Cases seen at University of California Hospitals and Clinics from 1918–47.

	11.	615	315	292	8	—	1. 08	—	New York, N.Y.	Pathologically proved cases of female breast cancer seen in Francis Delafield Hospital in New York City, 1952–58. Includes 479 whites, 136 Negroes.
	12.	3340	1333	1236	—	771	1. 08	—	Alabama	Cases in the Alabama Pathologists' Association Cancer Registry, 1948–55. Includes 2,643 whites, and 697 Negroes.
	13.	5322	1961	1815	—	1546	1. 08	Dorn & Cutler (*3*)	10 cities, U.S.A.	Morbidity Study in 10 metropolitan areas 1947–48: 4,876 white females and 446 nonwhite females included.
	14.	205	94	86	—	25	1. 09	Putzki & Scully (*18*)	Washington, D.C.	Private patients treated and followed by 3 physicians—dates not given.
	15.	203	104	94	5	—	1. 11	Robnett *et al.* (*19*)	Cleveland	Consecutive surgically treated patients over a 10-year period, Cleveland Clinic.
	16.	87	43	38	—	6	1. 13	Lee & Cornell (*20*)	New York, N.Y.	Patients (operable cases) admitted to New York Hospital prior to April 1919.
	17.	158	87	69	2	—	1. 26	Sims & Hamilton (*21*)	Columbus, Ohio	Consecutive cases seen at Mt. Carmel Hospital, Columbus, 1921–31.
	18.	298‡	175	129	—	—	1. 36	Finney *et al.* (*22*)	Baltimore, Md.	Consecutive cases seen in private practice; dates not given.
TOTAL		15, 931	6693	6465	86	2704	1. 04			

*Six bilateral cancers added to each side.
†Five bilateral cancers added to each side.
‡Six bilateral cancers added to each side.

in left/right occurrence of breast cancer, but the range of ratios from smallest to highest in foreign countries seems to be narrower than those in the various American series, even when only the larger series in the latter group are considered.

It is unfortunate that in many of the series shown in table 2, especially the Morbidity Study of 1947–48 and the cancer registry data, in a relatively high proportion of cases the side of breast affected was not given. We made some calculations by applying the ratio of the "known" left and right breast cancers to the "unknown" group of 2,724 cases. Based on these calculations, it seems doubtful that proper assignment of the "not stated" cases could seriously affect the over-all American ratio.

With respect to the occurrence of "bilateral" cancer of the breast, the figures given in tables 1 and 2 should not be taken too literally. Some authors choose to delegate sidedness to the first breast that is treated; others have assigned sidedness to both breasts. Still others have taken into consideration the elapsed time from the diagnosis of cancer in one breast to the occurrence of cancer in the other. Smithers, for example, found a total of 113 cases (6.4%) in which both breasts were involved. Of these, he deemed 60 as being recurrent in the second breast, and 53 as new primaries and therefore truly bilateral. Harrington (*23*) found an incidence of 3.4 percent in nonsimultaneous bilateral cancer and 1 percent for simultaneous.

COLOR AND REGION

Table 3 shows the ratios in the American data of left to right breast cancer, by color. We have included in this tabulation only the series in which side of breast was given in relation to color. For this investigation we made a special effort to obtain information on side of breast in cancer cases of colored women because a preliminary analysis indicated that differences in sidedness existed between white and colored cases. Data on colored cases from the New York State Cancer Registry and the M. D. Anderson Hospital in Houston have been added to make a total of 2,441 cases of breast cancer in colored women for whom side of breast was stated. In addition, Sidney Cutler, of the National Cancer Institute, furnished us with additional data, by side of breast, for colored women in the Morbidity Study, with respect to their age and the region of country.

The table shows that the over-all ratio for white women was 1.05 and for colored women, 1.03. This is not a significant difference ($P = 0.67$). When analyzed by region of the United States (North and South), the white women in the South had a ratio of 1.09 as compared with a ratio of 0.96 for Northern white women. This is a statistically significant difference ($P = 0.04$). Among the colored women, the ratios reversed. Northern colored women had a ratio of 1.24 as compared to a ratio of 0.97 for colored women in the South. This is a significant difference ($P = 0.01$).

Why these differences should exist, and why there should be a greater

TABLE 3.—Ratios of occurrence of left and of right breast cancer among American females, by color and region

	Total cases with side stated	Left breast	Right breast	Left/ right ratio	Source
					A) *White women:*
1.	538	261	277	0. 94	Tumor Clinic, University of Minnesota Hospitals
2.	931	455	476	0. 96	Cancer Registry, Westchester Co., N.Y.
3.	471	235	236	1. 00	Francis Delafield Hospital, New York, N.Y.
4.	365	183	182	1. 01	Cancer Registry, Charity Hospital, New Orleans, La.
5.	3477	1805	1672	1. 09	Morbidity Study in 10 metropolitan areas
6.	2029	1067	962	1. 11	Cancer Registry, Alabama Pathologists' Association
TOTAL	7811	4006	3805	1. 05	
					B) *Colored women:*
1.	619	293	326	0. 90	Cancer Registry, Charity Hospital, New Orleans, La.
2.	540	266	274	0. 97	Cancer Registry, Alabama Pathologists' Association
3.	363	183	180	1. 02	Cases from M. D. Anderson Hospital, Houston, Tex., 1944–58
4.	187	95	92	1. 03	Meharry Medical College, Nashville, Tenn.
5.	50	26	24	1. 08	Cancer Registry, Westchester Co., N.Y.
6.	299	156	143	1. 09	Morbidity Study in 10 metropolitan areas
7.	247	141	106	1. 33	Cancer Registry, New York State, 1940–54
8.	136	80	56	1. 43	Francis Delafield Hospital, New York, N.Y.
TOTAL	2441	1240	1201	1. 03	
					C) *Color and region:**
1.	1940	951	989	0. 96	White women—North
2.	2394	1250	1144	1. 09	White women—South
3.	618	347	281	1. 24	Colored women—North
4.	1813	893	920	0. 97	Colored women—South

*White women in Dorn's Morbidity Study are not included in this table. Data on colored women by region are included.

ratio for Southern white women and for Northern colored women is puzzling. It could be a function of the selection of the series used in this analysis. To verify that these differences are real, it would be desirable to further investigate these findings with more cases.

AGE

Clemmesen (*24*) and Dorn and Cutler have noted a break in the rising incidence curve for breast cancer around the age of 45 to 54, although MacMahon (*25*) has proposed that this break may be explained by different changes in the incidence rates for cohorts of women born in different years. For our data, we grouped our cases by broad age groups to determine whether any of the observed differences in sidedness could be

explained by the age distribution of the various cases. Table 4 shows the ratios for side of breast by age, broken down by color for all the groups of cases for which we had age data. Even using the broad age groups we set, there is a good deal of fluctuation in the ratios. No consistent pattern for the menopausal age group, 45 to 54 years, could be seen, although it may be remarked that Busk and Clemmesen did find more right than left breast cancer in single women in age group 45 to 54.

The one feature that does emerge is the lower left/right ratio for age group 70 and over. This is presented in summary form in table 5. In

TABLE 4.—Ratios of occurrence of left and of right breast cancer among females, by age and color

Age	Left breast	Right breast	Left/right ratio	Left breast	Right breast	Left/right ratio	Left breast	Right breast	Left/right ratio
	Westchester—white			Westchester—colored			Francis Delafield—white		
Under 30	5	4	1. 25	—	—	—	2	3	0. 67
30–44	90	82	1. 10	4	8	0. 50	52	43	1. 21
45–54	118	118	1. 00	5	3	1. 67	68	67	1. 01
55–69	157	159	0. 99	15	7	2. 15	87	96	0. 91
70+	85	112	0. 76	2	6	0. 33	26	27	0. 96
Age not stated	—	1	—	—	—	—	—	—	—
TOTAL	455	476	0. 96	26	24	1. 08	235	236	1. 00
	Francis Delafield—colored			Cancer Reg., N.Y.—colored			Morbidity Study North—colored		
Under 30	7	1	7. 00	3	3	1. 00	2	3	0. 67
30–44	21	20	1. 05	45	32	1. 41	31	36	0. 86
45–54	20	18	1. 11	39	31	1. 26	28	17	1. 65
55–69	22	10	2. 20	41	24	1. 71	31	27	1. 15
70+	10	7	1. 43	13	16	0. 81	8	12	0. 67
Age not stated	—	—	—	—	—	—	—	—	—
TOTAL	80	56	1. 43	141	106	1. 33	100	95	1. 05
	Morbidity Study South—colored			Alabama—white			Alabama—colored		
Under 30	—	—	—	23	28	0. 82	9	6	1. 50
30–44	18	11	1. 64	274	250	1. 10	80	76	1. 05
45–54	19	12	1. 58	277	244	1. 14	71	82	0. 87
55–69	15	16	0. 94	347	295	1. 18	69	76	0. 91
70+	4	8	0. 50	128	122	1. 05	34	31	1. 10
Age not stated	—	—	—	18	23	—	3	3	—
TOTAL	56	48	1. 17	1067	962	1. 11	266	274	0. 97
	Charity Hosp., New Orleans—white			Charity Hosp., New Orleans—colored			Meharry Med. Coll.—colored		
Under 30	6	5	1. 20	3	9	0. 33	3	2	1. 50
30–44	27	34	0. 79	85	86	0. 99	18	18	1. 00
45–54	40	40	1. 00	76	84	0. 90	26	33	0. 79
55–69	71	64	1. 11	100	93	1. 08	27	28	0. 96
70+	39	39	1. 00	29	54	0. 54	18	7	2. 57
Age not stated	—	—	—	—	—	—	3	4	—
TOTAL	183	182	1. 01	293	326	0. 90	95	92	1. 03

TABLE 5.—Summary of ratios of occurrence of left and of right breast cancer by age under 70 years, and over 70, in Denmark, and among American females, by color and region

Age	Left breast	Right breast	Left/right ratio	Left breast	Right breast	Left/right ratio
	White females—North			White females—South		
Under 70	579	572	1. 01	1065	960	1. 11
70+	111	139	0. 80	167	161	1. 04
TOTAL	690	711	0. 97	1232	1121	1. 10
	Colored females—North			Colored females—South		
Under 70	314	240	1. 31	619	632	0. 98
70+	33	41	0. 80	85	100	0. 85
TOTAL	347	281	1. 23	704	732	0. 96
	Busk & Clemmesen—Denmark					
Under 70	1781	1566	1. 14			
70+	336	342	0. 98			
TOTAL	2117	1908	1. 11			

each of the grouped cases by color and region, and for Busk and Clemmesen's data, there is a lower left/right ratio in those 70 years and over compared with those up to the age of 70 years. In fact, there appears to be a reversal in the left/right ratio. These differences are more pronounced for the cases obtained in the North (both white and colored) and in Busk and Clemmesen's series than in the South. Except for the Northern colored cases, which are barely significant at 0.05 level, none of these groups show a significant difference. The fact that the lower ratio does exist for 70 years and over in all these groups would seem to indicate that factors associated with old age could be of importance and should be further investigated with more cases.

MARITAL STATUS

There is a substantial body of data accumulated to show that single women have death rates higher than married women, and that married women without children have death rates higher than those who have had children. Busk and Clemmesen have presented data by side of breast and by age for single women and for married, widowed, and divorced combined. As mentioned previously, their data show a greater occurrence of breast cancer in the right breast than in the left for single women 45 to 54 years of age. This reversal does not hold in the ever-married women group. Moreover, the reversed left/right ratio for those 70 years and over holds for the latter group, but not for the single women. Regardless of age, the over-all left/right ratios for their data show a 1.05 ratio for single women and a 1.12 ratio for the married, widowed, and divorced group as a whole.

Our data from three series suggest that widowed, divorced, and separated women have higher left/right ratios than either the single or the

married. Grouping marital status according to Busk and Clemmesen, the single women had a ratio of 1.11, while the ever-married women had a ratio of 1.15.

The number of cases by marital status in our data are too few to analyze by age. It would appear, however, that there may be differences in sidedness by marital status. This is worthy of further investigation with more cases.

STAGE

We made an attempt to determine sidedness by stage of disease, but this investigation had several difficulties. Our cases were classified by a broad clinical staging of localized, regional involvement and remote metastasis. No striking differences were noted in this analysis; besides, there was a high percentage with stage not stated. Moreover, in the discussion of staging of breast cancer, in the literature, many different methods have been presented (Portmann's, Steinthal's, and Haagensen-Stout's) all of which would probably be more suitable for insight in analysis of sidedness in breast cancer. Shimkin (*17*) used the classification method proposed by Portmann and found the following left/right ratios:

	Left breast	Right breast	Left/right ratio
Stage I	163	171	.0. 95
Stage II	148	151	0. 98
Stage III	178	135	1. 32
Stage IV	55	52	1. 06
Total	544	509	1. 07

In this one series, it may be seen that the excess of left breast-cancer cases is concentrated in the later stages, particularly Stage III.

MALE BREAST CANCER

Table 6 shows the occurrence, by side of breast, for males. Even with the small number of cases, the over-all ratio of 1.33 is significantly higher than an equal distribution by side ($P = 0.01$). The left-to-right excess in the American males is significantly different from that of the American females ($P = 0.02$). The limited English experience for males shows virtually the same left/right ratio as for females.

DISCUSSION

In 1926 Lane-Claypon (*27*), in a study of antecedent factors in breast cancer, found a higher incidence of history of injury in breast-cancer cases than in a control series. In the breast-cancer cases, 59.6 percent of all instances of injury were in the left breast. Unfortunately, the data

TABLE 6.—Ratios of occurrence of left and of right breast cancer among males

	Total cases	Left breast	Right breast	Bilateral	Not stated	Left/right ratio	Source	Location	Description of cases
1.	23	11	12	—	—	0.91	Hartnett (*5*)	England	All hospitals in Administering County of London, 1938–39.
2.	70	33	30	—	7	1.10	Registrar-General (*9*)	England & Wales	Deaths from cancer of the breast, in England and Wales, 1931–34
3.	146	82	63	1	—	1.30	Treves & Holleb (*26*)	New York, N.Y.	Clinically diagnosed cases seen in Memorial Hospital, New York, N.Y., 1924–54
4.	31	17	11	—	3	1.55	—	Alabama	Cases in the Alabama Pathologists' Association Cancer Registry, 1948–55
5.	13	7	4	—	2	1.75	—	Westchester Co., N.Y.	Cases reported to the Central Cancer Registry in Westchester County, 1948–58
6.	15	9	5	1	—	1.80	Smithers *et al.* (*2*)	England	Cases seen in Royal Cancer Hospital, London, 1937–48
7.	49	21	10	—	18	2.10	Dorn & Cutler (*3*)	10 cities, U.S.A.	Morbidity Study in 10 metropolitan areas, 1947–48
TOTAL	347	180	135	2	30	1.33			
SUBTOTAL—English experience	108	53	47	1	7	1.13			
SUBTOTAL—American experience	239	127	88	1	23	1.44			

do not include the side of breast for those cancer cases in which there was no history of bruising. In reviewing Lane-Claypon's work, Smithers made calculations on the hypothesis that for cancer cases without injury, in her series, there were an equal number of left and of right breast cacers. On this basis Smithers found that for the total series, breast cancer occurred 11 percent more often in the left than in the right breast. This paralleled the findings on the occurrence of left and right breast cancer in his own cases.

Smithers was hesitant to place any weight on this analysis. Can the excess of left over right breast cancer be attributable to trauma? There have been breast-cancer cases reported in women that were directly attributable to trauma. In male breast-cancer cases, trauma appears to be associated with the development of cancer more often than in women. Treves and Holleb (*26*) found that 12 percent of their male breast-cancer cases gave a history of trauma. Because of a wide variety of types and frequency of trauma, and range from onset of trauma to breast-cancer symptoms, they felt that no relationship existed. Charache (*28*) reviewed the literature and found a history of trauma existed in 25 percent of male breast cancers.

If trauma is an etiological factor in the development of breast cancer and through some mechanism occurs more frequently in the left breast than in the right breast, then it is plausible that in the general population there should more often be injury to the left than to the right breast. In September of 1958, a questionnaire was mailed to a random sample of 5,000 persons throughout the country, as a pretest of a questionnaire, designed to be used by the American Cancer Society in a large-scale prospective study of factors relating to cancer. We included in this questionnaire one on injury to the breast. Of 1,005 women who replied (about 40% of all women), 26 said that they had had an injury to the breast sometime in their lives. Of these, 13 injuries were to the left breast, 9 to the right breast, 2 to both breasts, and 2 women did not state which breast. In Lane-Claypon's study, 28 or 5.5 percent of the 509 women in her non-cancer group reported having a breast injury, with 12 reporting the trauma to the left breast, 8 to the right, 1 in both, and 7 did not specify the side. These results are, of course, inconclusive, but they are interesting in that they are consistent with the theory that the left breast is more injury-prone.

Segi *et al.* (*10*) reported, for breast-cancer cases and controls, the frequency of disturbance of milk secretion, by side of breast, among women who had had children, using the lactation period after each childbirth as an enumeration unit. He found that:

1. In the controls there was a greater frequency of poor milk secretion in the left breast than in the right breast.
2. Poor milk secretion in the left breast was much more frequent in left breast-cancer cases than in controls; similarly, poor milk secretion in the right breast was more frequent in right breast-cancer cases than in controls. At the same time, poor milk secretion in the

breast not affected by cancer was only slightly greater for breast-cancer cases than for controls.

Further inspection of Segi's data reveals that among women with a history of poor milk secretion of the left side only, 83 percent of the breast cancers were of the left breast; while of those with poor milk secretion of the right side only, 60 percent of the breast cancers were of the right side. From his analysis Segi concludes, "For finding out the cause of why cancer of the breast is more frequent on the left side, it seems we should first make it clear why dyslactation of the left breast is more frequent than that of the right."

While Segi's findings are of great value for the study of the development of breast cancer, it should be noted that the development of breast cancer associated with poor milk secretion can only account for a minor proportion of the total left/right difference. Crude calculations based upon Segi's data show that about 8 percent of women with left breast cancer had poor lactation in the left breast only; and that about 5 percent of the women with right breast cancer had poor lactation in the right breast only. In terms of the total left/right ratio in the Japanese data, the association of poor milk secretion with the development of cancer can only account for perhaps one fifth of the difference.

To advance the understanding of the left/right difference, a thorough study of the physiological and pathological differences in the breasts would be most desirable. Deaton and Bradshaw (*29*) have suggested that aberrant breast tissue occurs frequently in the upper outer quadrant of the breast and has a tendency to develop into breast cancer, and that aberrant breast tissue occurs more frequently in the left than in the right breast. This is a further possible theory to explain the predominance of left breast cancer.

Any theory of differences in the breasts has to be examined in the light of the existing differences in the American and foreign experience outlined. In addition, the apparent difference in left/right ratios of women under 70 years and over 70 has to be considered. The difference in the American and foreign ratios cannot be explained by the age factor alone. For example, in the series of 1,205 cases obtained from the Charity Hospital Cancer Registry in New Orleans, with a ratio of 0.94, 16 percent of the women were over the age of 70; in Busk and Clemmesen's Danish series, with a left/right ratio of 1.11, 17 percent, virtually the same proportion were over the age of 70.

One other possible area of investigation to explain the left/right difference concerns the physical size of the left breast as compared to the right. It is conceivable that the greater occurrence of left breast cancer is attributable to the fact that in the general population there is more breast tissue in the left than the right breast. It would be admittedly difficult to determine how the breasts should be measured, but even a small-scale study designed to test this hypothesis would be interesting.

From this study, it is evident that many fruitful areas of investigation into cancer may be made by studying the laterality of occurrence of

do not include the side of breast for those cancer cases in which there was no history of bruising. In reviewing Lane-Claypon's work, Smithers made calculations on the hypothesis that for cancer cases without injury, in her series, there were an equal number of left and of right breast cacers. On this basis Smithers found that for the total series, breast cancer occurred 11 percent more often in the left than in the right breast. This paralleled the findings on the occurrence of left and right breast cancer in his own cases.

Smithers was hesitant to place any weight on this analysis. Can the excess of left over right breast cancer be attributable to trauma? There have been breast-cancer cases reported in women that were directly attributable to trauma. In male breast-cancer cases, trauma appears to be associated with the development of cancer more often than in women. Treves and Holleb (*26*) found that 12 percent of their male breast-cancer cases gave a history of trauma. Because of a wide variety of types and frequency of trauma, and range from onset of trauma to breast-cancer symptoms, they felt that no relationship existed. Charache (*28*) reviewed the literature and found a history of trauma existed in 25 percent of male breast cancers.

If trauma is an etiological factor in the development of breast cancer and through some mechanism occurs more frequently in the left breast than in the right breast, then it is plausible that in the general population there should more often be injury to the left than to the right breast. In September of 1958, a questionnaire was mailed to a random sample of 5,000 persons throughout the country, as a pretest of a questionnaire, designed to be used by the American Cancer Society in a large-scale prospective study of factors relating to cancer. We included in this questionnaire one on injury to the breast. Of 1,005 women who replied (about 40% of all women), 26 said that they had had an injury to the breast sometime in their lives. Of these, 13 injuries were to the left breast, 9 to the right breast, 2 to both breasts, and 2 women did not state which breast. In Lane-Claypon's study, 28 or 5.5 percent of the 509 women in her non-cancer group reported having a breast injury, with 12 reporting the trauma to the left breast, 8 to the right, 1 in both, and 7 did not specify the side. These results are, of course, inconclusive, but they are interesting in that they are consistent with the theory that the left breast is more injury-prone.

Segi *et al.* (*10*) reported, for breast-cancer cases and controls, the frequency of disturbance of milk secretion, by side of breast, among women who had had children, using the lactation period after each childbirth as an enumeration unit. He found that:

1. In the controls there was a greater frequency of poor milk secretion in the left breast than in the right breast.
2. Poor milk secretion in the left breast was much more frequent in left breast-cancer cases than in controls; similarly, poor milk secretion in the right breast was more frequent in right breast-cancer cases than in controls. At the same time, poor milk secretion in the

breast not affected by cancer was only slightly greater for breast-cancer cases than for controls.

Further inspection of Segi's data reveals that among women with a history of poor milk secretion of the left side only, 83 percent of the breast cancers were of the left breast; while of those with poor milk secretion of the right side only, 60 percent of the breast cancers were of the right side. From his analysis Segi concludes, "For finding out the cause of why cancer of the breast is more frequent on the left side, it seems we should first make it clear why dyslactation of the left breast is more frequent than that of the right."

While Segi's findings are of great value for the study of the development of breast cancer, it should be noted that the development of breast cancer associated with poor milk secretion can only account for a minor proportion of the total left/right difference. Crude calculations based upon Segi's data show that about 8 percent of women with left breast cancer had poor lactation in the left breast only; and that about 5 percent of the women with right breast cancer had poor lactation in the right breast only. In terms of the total left/right ratio in the Japanese data, the association of poor milk secretion with the development of cancer can only account for perhaps one fifth of the difference.

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From this study, it is evident that many fruitful areas of investigation into cancer may be made by studying the laterality of occurrence of

malignancy. This applies not only to the breast, but to other organs such as the kidney, the bladder, and the ovary. The lung and the bronchus may perhaps also be studied with respect to laterality, but here the fact that the right lung has one more lobe than the left might make a comparison by side difficult. In reviewing the literature for this paper, we found that an abortive attempt was made by the Registrar-General of England and Wales in 1931–34 to study sidedness in these organs in addition to side of breast, but there were so many cases where side was not specified for the lung, the kidney, the bladder, and the ovary that not much could be said of the occurrence of left/right cancer for these organs. Perhaps an approach to the study of sidedness of cancer is to supplement the International List of Diseases and Causes of Death to include a fourth digit for side of organ involved. This may alert physicians to record more specifically the side affected in these organs, in morbidity and mortality data.

REFERENCES

(1) Busk, T., and Clemmesen, J.: The frequencies of left- and right-sided breast cancer. Brit. J. Cancer 1: 345–351, 1947.

(2) Smithers, D. W., Rigby-Jones, P., Galton, D. A. G., and Payne, P. M.: Cancer of the breast. A review. Brit. J. Radiol. (Supp. 4), pp. i–xiii; 1–90, 1952.

(3) Dorn, H. F., and Cutler, S. J.: Morbidity from Cancer in the United States. U.S. Department of Health, Education, and Welfare, Public Health Service, Pub. Health Mono. No. 29, 1955.

(4) Nohrman, B. A.: Cancer of the breast; a clinical study of 1,042 cases treated at Radiumhemmet, 1936–1941. Acta Radiologica (Supp. 77), pp. 1–98, 1949.

(5) Hartnett, W. L.: A statistical report on 2,529 cases of cancer of the breast. Brit. J. Cancer 2: 212–239, 1948.

(6) Valderrama, J. A. J., Munoz, J. J., and Laverde, H. F.: Cancer del seno: consideraciones clinicas, tratamiento y resultados sobre 486 casos. Rev. Colombi. Cancer., No. 1, 1954, pp. 95–127.

(7) Ballarati, U.: Cancro della mammella; considerazioni statistiche e risultati lontani. Tumori 38: 289–312, 1952.

(8) von Fellenberg, R.: Swiss statistics on malignant tumors. Bull. Eidgenoss. Gesundheitsamt, Bern, 1940.

(9) Registrar-General's Statistical Review of England and Wales for the Year 1935. Her Majesty's Stationery Office, London.

(10) Segi, M., Fukushima, I., Fujisaku, S., Kurihara, M., Saito, S., Asano, K., and Kamoi, M.: An epidemiological study of cancer in Japan. Gann Japanese J. Cancer Res., Vol. 48, Supp., 1957.

(11) Deaton, W. R., Jr., Postlethwait, R. W., and Bradshaw, H. H.: Carcinoma of the breast, with analysis of 134 cases. North Carolina M. J. 10: 484–488, 1949.

(12) Antonellis, C. J.: Carcinoma of the breast. West Virginia M. J. 50: 178–183, 1954.

(13) Kaplan, I. I., and Rosh, R.: Cancer of the breast. A study of patients treated over a period of 20 years in the Radiation Therapy Department of Bellevue Hospital, New York City. Radiology 47: 583–592, 1946.

(14) Siris, E., and Dobson, L.: Analysis of 402 cases of carcinoma of the breast. California Med. 65: 201–206, 1946.

(*15*) KENNEDY, A. T., and JELKS, E.: Cancer of the breast; a study of 100 cases at the Duval County Hospital. J. Florida M.A. 34: 343–346, 1947.

(*16*) ANDERSON, V. E., GOODMAN, H. O., and REED, S. C.: Variables Related to Human Breast Cancer. Minneapolis, Univ. Minnesota Press, 1958.

(*17*) SHIMKIN, M. B., LUCIA, E. L., STONE, R. S., and BELL, H. G.: Cancer of the breast—analysis of frequency, distribution, and mortality at the University of California Hospital, 1918 to 1947, inclusive. Surg., Gynec. & Obst. 94: 645–661, 1952.

(*18*) PUTZKI, P. S., and SCULLY, J. H.: Two hundred and five cases of cancer of breast treated by radical mastectomy. Surg., Gynec. & Obst. 83: 751–760, 1946.

(*19*) ROBNETT, A. H., JONES, T. E., and HAZARD, J. B.: Carcinoma of the breast, recurrence and survival in 203 patients. Cancer 3: 757–772, 1950.

(*20*) LEE, B. J., and CORNELL, N. W.: A report of 87 primary operable cases of carcinoma of the breast admitted to New York Hospital prior to April 1, 1919. Am. Surg. 80: 400–418, 1924.

(*21*) SIMS, G. P., and HAMILTON, C. S.: A study of 158 consecutive cases of breast malignancy. Ohio State M. J. 32: 406–410, 1936.

(*22*) FINNEY, G. G., MERKEL, W. C., and MILLER, D. B.: Carcinoma of the breast; a study of 298 consecutive cases. Ann. Surg. 125: 673–687, 1947.

(*23*) HARRINGTON, S. W.: Survival rates of radical mastectomy for unilateral and bilateral carcinoma of breast. Surgery 19: 154–166, 1946.

(*24*) CLEMMESEN, J.: Carcinoma of the breast; symposium; results from statistical research. Brit. J. Radiol. 21: 583–590, 1948.

(*25*) MACMAHON, B.: Breast cancer at menopausal ages: an explanation of observed incidence changes. Cancer 10: 1037–1044, 1957.

(*26*) TREVES, N., and HOLLEB, A. I.: Cancer of the male breast; a report of 146 cases. Cancer 8: 1239–1250, 1955.

(*27*) LANE-CLAYPON, J. E.: A Further Report on Cancer of the Breast, with Special Reference to Its Associated Antecedent Conditions. Reports on Public Health and Medical Subjects, No. 32, Ministry of Health, London, 1926.

(*28*) CHARACHE, H.: Tumors of the male breast. Surgery 7: 889–899, 1940.

(*29*) DEATON, W. R., JR., and BRADSHAW, H. H.: Carcinoma of the breast—why the upper outer quadrant? Surgery 20: 583–584, 1950.

Investigation of Tumor Variants Recovered From Mice With Systemic Leukemia (L1210) After Extensive Therapy With 3′,5′-Dichloroamethopterin and 3′-Bromo-5′-Chloroamethopterin [1, 2]

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SUMMARY

Studies were conducted on the growth behavior and sensitivity to therapy of tumors recovered from mice with systemic leukemia (L1210) after a prolonged increase in their lifespan, by treatment with 3′,5′-dichloroamethopterin and 3′-bromo-5′-chloroamethopterin. The tumors were recovered from various sites including the spleen and the blood. They were histologically similar to the original L1210, but showed a wide range of growth rates and sensitivities to treatment. Some of the recovered tumors grew and killed rapidly like the original sensitive leukemia L1210. Others grew slowly, and the mice showed prolonged survival times, the local tumor at the subcutaneous site of injection becoming quite large (20–35 mm.) prior to death. Some of the tumors retained the original sensitivity to treatment with amethopterin, while others showed a high degree of resistance. Resistant variants resulting from treatment with 3′,5′-dichloroamethopterin, or with amethopterin, showed greater sensitivity to 3′,5′-dichloroamethopterin than to amethopterin. 6-Mercaptopurine retained its effectiveness against the resistant variants. These observations are discussed with respect to therapy.—J. Nat. Cancer Inst. 23: 633–653, 1959.

SINCE THE demonstration by Farber (*1*) of the clinical usefulness of aminopterin in the treatment of children with acute leukemia, there has been continued interest in investigation of folic acid antagonists. Two of the dihalogenated derivatives of amethopterin, 3′,5′-dichloroamethopterin and 3′-bromo-5′-chloroamethopterin, were recently demonstrated to be capable of producing extensive increases in the survival time of mice with systemic leukemia, L1210 sensitive, (*2–5*). When daily treatment with either drug was initiated only a few days prior to the death of untreated leukemic mice, survival times for the full period of treatment (up to 90 days post leukemic inoculation) and beyond were obtained. Some of the mice showed no evidence of disease after 6 months and presumably were cured (*5*).

[1] Received for publication March 6, 1959.
[2] Presented in part at the Fiftieth Annual Meeting of the American Association for Cancer Research, April 10–12, 1959.
[3] With the technical assistance of Gerald O. Chapman and Gerome McDowell.
[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

In the assay system employed for systemic leukemia, treatment was initiated when a local tumor, 8 to 12 mm. in diameter, was present at the site of leukemic inoculation (*6*). With optimal doses of the dihalogenated derivatives of amethopterin, the local tumor usually regressed after several treatments, and did not reappear either during the course of treatment or subsequent to the eventual discontinuance of therapy.

However, regression of the local tumor does not signify that the disease has been wholly eradicated (*5*). During extensive therapy, or subsequent to the discontinuance of therapy, mice showed tumor growth in areas other than that of initial implantation. Palpable tumors appeared at various sites. Some of the mice developed tumors in the maxillary area. In a few instances tumor growth was observed in one of the eye sockets. Solid internal tumors were also seen, in association with the liver, kidney, and other organs. Also, during or after the discontinuance of long-term therapy, some of the animals that no longer showed evidence of a palpable tumor and were in an apparently healthy condition for an extended period of time, subsequently became moribund, lost weight, and died. On autopsy the spleen was not necessarily enlarged, and frequently there was no other gross evidence of tumor. When such moribund animals were killed, transplantation of a spleen mash invariably resulted in tumor growth. Transplantation of blood at this time also resulted in tumor growth in most of the mice.

The current report describes the growth behavior and responsiveness to therapy of tumors recovered from mice with systemic leukemia (L1210), during or subsequent to extensive therapy with 3′,5′-dichloroamethopterin or 3′-bromo-5′-chloroamethopterin. Comparison is made with the parent sensitive L1210 and with an amethopterin-resistant line of L1210 carried in this laboratory.

METHODS

The general procedures have been described previously (*2*, *6–8*). The experiments were conducted with (BALB/cAn × DBA/2J)F_1 hybrid male mice, unless otherwise indicated. The tumors tested in the current study were derived from mice with advanced lymphoid leukemia (L1210 sensitive) that had been treated for an extensive period with either 3′,5′-dichloroamethopterin or 3′-bromo-5′-chloroamethopterin. The tumors recovered from mice treated with 3′,5′-dichloroamethopterin are from experiments S207 (*9*) and J320 (*5*). The tumors of mice treated with 3′-bromo-5′-chloroamethopterin are from various groups in experiment J325 (*5*).

A listing and history of the tumors tested in the current study are shown in table 1. In the original experiments from which the tumors were obtained, treatment had been initiated on the 7th or 8th day after leukemic inoculation and continued daily until the 90th day, unless the animals were killed earlier. The mice were killed from 71 to 200 days

from the time of leukemic inoculation. The source of tumor material, after the above treatments, was the spleen, the solid tumor, or the blood.

Comparisons are made with two lines of the sensitive L1210 (*10*), the Z line carried in the indigenous strain (DBA/2J) and the BB line carried in (BALB/cAn × DBA/2J)F_1 hybrids. The amethopterin-resistant variant of L1210 (M46R) was obtained by treatment with 3 mg. per kg. of amethopterin on alternate days in serial intraperitoneal passage of the sensitive leukemia (L1210–Z) in the hybrid mice. It had been carried subcutaneously for 22 generations without treatment when employed in the current study.

A suspension of spleen cells or tumor cells was employed as the inoculum in the experiments in which the tumors were tested for sensitivity to therapy with amethopterin, 3′,5′-dichloroamethopterin, or 6-mercaptopurine. The cell suspensions were inoculated subcutaneously into the right hind leg (0.1 ml. of leukemic cell suspension per mouse). The inoculum (approximately 500,000 cells per mouse) was generally 10 to 100 times greater than that required to give 100 percent mortality in a serial dilution of the inoculum in controls. Treatment was initiated 2 to 3 days after leukemic inoculation, or, when indicated, after the appearance of a local tumor at the site of leukemic inoculation. Amethopterin and 3′,5′-dichloroamethopterin [5] were dissolved in a 2 percent solution of sodium bicarbonate. 6-Mercaptopurine [6] was dissolved in dilute sodium hydroxide. The drugs were administered subcutaneously in the axillary region at 0.01 ml. per gm. of body weight. Treatment was given daily until all the animals succumbed. The mice were observed for mortality, and observations were made of local tumor size and body weight.

Tissues were fixed in Zenker-formol and stained with hematoxylin and eosin.

RESULTS

The data are summarized in tables 1 through 6 and text-figures 1 through 4. Table 1 lists the derived tumors and shows the history of the original experiments with 3′,5′-dichloroamethopterin and 3′-bromo-5′-chloroamethopterin from which the various tumors were recovered.

Summary data are also presented (table 1) of the experiments in which the tumors were tested for sensitivity to treatment with amethopterin. The generation number and the source of the experimental inoculum are designated for each test. Also included are the median survival time of controls, the optimal daily dose for amethopterin, and the median survival time at the optimal dose. The relative increase in median survival time elicited at the optimal dose of amethopterin as compared with the

[5] The folic acid analogues were provided by the Research Division, American Cyanamid Company, Pearl River, N.Y.

[6] 6-Mercaptopurine was provided by Dr. George Hitchings, of the Burroughs Wellcome Research Laboratories, Tuckahoe, N.Y.

TABLE 1.—History and sensitivity to amethopterin of tumors recovered from mice with advanced leukemia (L1210) after prolonged treatment with halogenated derivatives of amethopterin

Derived tumor	History of mice with advanced leukemia (L1210) from which tumors derived					Tests of sensitivity to amethopterin						
Expt.–group No.*	Daily dose (*mg./kg.*)	Days treated	Day of sacrifice	Source of derived tumor	Median survival time of group	Expt. No.	Tumor generation	Source of expt. inoculum	Median survival time of controls	Optimal daily dose (*mg./kg.*)†	Median survival time at optimal dose	Relative median survival time: Controls = 1
	3′,5′-Dichloro-amethopterin											
S207–2_4	75	7–90	91	Tumor‡	111	C44	2	Spleen	14	0.78;1.30	19	1.36
S207–2_5	75	7–90	—	Tail blood	111	C45	1	Spleen	9.5	1.29	17.5	1.85
						C46	6	Spleen	10.5	0.8;1.0;1.56	20	1.90
						C58	16	Spleen	9.0	2.0	28.5	3.17
J320–$3A_7$	28	8–60	86	Spleen	32.5	S200	2	Tumor	36	1§	37	1.03
	3′-Bromo-5′-chloro-amethopterin											
J325–$4A_2$	129	8–90	106	Spleen	74.5	C45	2	Spleen	10	1.29	34	3.40
J325–$4A_3$	129	8–90	106	Spleen	74.5	C51	3	Spleen	37.5	1§	63	1.69
J325–$4A_4$	129	8–90	96	Spleen	74.5							
J325–$4A_9$	129	8–90	200	Spleen	74.5							
J325–$4B_{10}$	77.2	8–90	109	Spleen	54.5							
J325–$4C_2$	46.3	8–72	72	Spleen	60.5	C40	2	Spleen	12	0.47	19.5	1.63
J325–$4C_9$	46.3	8–90	96	Spleen	60.5	C45	2	Spleen	66.5	1.29	93.5	1.40
J325–$4E_5$	16.7	8–90	91	Tumor‖	34	C44	1	Tumor‖	41	1.29	73	1.78
J325–$4H_5$	3.6	8–71	71	Spleen	27.5	C40	3	Spleen	12.5	1.30	34	2.75
	Controls—sensitive L1210**											
Expt. S207					11	C40	209	Spleen	11	1.30	40.5	3.68
Expt. J320					11	C44	211	Spleen	10	1.29	39	3.90
Expt. J325					10	C45	212	Spleen	13	1.29	64.5	4.96
						C51	232	Spleen	9	1	25	2.78
						C58	228	Spleen	9	2.0	35	3.82

*The letter and number preceding the dash indicate the number of the original experiment. The number (and letter where shown) following the dash designates the treatment group at the designated dosage. The subnumber designates the number of the individual mouse from which the tumor was derived.

†Treatment initiated at following days after tumor implantation: Day 2—expt. C40, C45; day 3—expt. C44, C46, C58; day 7—expt. C51; day 17—expt. S200.

‡The S207–2_4 tumor appeared subcutaneously in the left abdominal area.

§No attempt made to determine the optimal dose.

‖This tumor appeared in the maxillary area.

**BB subline in expt. S207, J320, J325, C51; Z subline in expt. C40, C44, C45, C58.

controls is shown in the last column. Data are also included on the two lines of the sensitive L1210 (Z and BB).

It may be seen in table 1 that the untreated mice inoculated with the various tumors recovered from animals that had been given prolonged treatment showed a wide range of median survival times (9–66.5 days for the S207–2_5 tumor and the J325–$4C_9$ tumor, respectively). The L1210 sensitive lines (Z and BB), in contrast, in five experiments showed uniform median survival times of short duration (9–13 days).

It may also be noted in table 1 that the recovered tumors showed a wide range of sensitivity to amethopterin. The relative median survival time for the treated tumorous mice, as compared with their own controls taken as unity, ranged from 1.03 (J320–$3A_7$ tumor), indicating marked resistance, to 3.40 (J325–$4A_2$ tumor), indicating definite sensitivity. With the original sensitive lines of L1210, the relative median survival times for the amethopterin-treated mice were 2.78 to 4.96 times greater than their controls.

Table 2 shows the survival time and tumor-size data for mice receiving uniform trocar implants (approximately 25 mg. each) of solid tumor, for 10 of the recovered tumors. Similar data are also shown with trocar implants of solid tumor of one of the original sensitive lines and with a serial dilution of leukemic cell suspension of the sensitive line. There was a wide range in survival time and in tumor size, prior to death, among the various recovered tumors. Tumor growth and survival time were similar to those of the sensitive line, with only 4 of the 10 tumors tested. The extensive survival time and large tumor diameters observed with 6 of the 10 derived tumors were not duplicated by serial dilution of the inoculum for the sensitive leukemia L1210–Z (table 2).

Microscopic sections of the various sublines of recovered tumors were examined for comparison with the original sensitive lines of leukemia L1210. Examination of the sections, stained with hematoxylin and eosin, did not reveal any significant differences between the derived tumors and the original leukemia L1210.[7]

Tumor J320–$3A_7$

Table 3 presents detailed data on the J320–$3A_7$ tumor—J320 is the experiment number from which the original mouse came. The group number is 3A, and the subnumber 7 indicates the number of the original mouse. The original mouse had received 28 mg. per kg. of 3′,5′-dichloroamethopterin daily from day 8 to day 60 and was killed on day 86. Table 3–A summarizes the history of the original mouse. The local tumor at the site of leukemic inoculation (originally 10 mm. in diameter) regressed, and the animal gained weight during the period of therapy. After the discontinuance of therapy, the mouse lost weight rapidly. On autopsy, at day 86, there was no gross evidence of tumor except for suspicious enlarged nodules in the submaxillary area.

[7] The authors wish to thank Dr. G. Burroughs Mider for examination of the sections.

TABLE 2.—Tumor growth and survival time for mice with trocar implants* of 10 of the sen

Tumor	Generation No.	Survival times after (*Days*) 7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Trocar																		
$S207–2_4$	17			7	2	1												
$S207–2_5$	19		4	4	1		1											
$J320–3A_7$	15																	1
$J325–4A_2$	19			4	5	1												
$J325–4A_4$	6															1		
$J325–4A_9$	6								2	2	1		1	2	1		1	
$J325–4B_{10}$	7																	
$J325–4C_2$	20			7	1	2												
$J325–4C_9$	7																	
$J325–4B_5$	7																	
Sensitive L1210–Z	232		4	6														
Cell suspension; sensitive L1210–Z																		
Expt. inoculum‡	209–212			5	7	8	4	3	3									
1/10	" "				2	2	1	1	1		1		1					
1/100	" "						2	1	2	1							§1	
1/1000	" "								1	1		1						

*The trocar implants weighed approximately 25 mg. each.
†Average of 2 dimensions.

The history of the first generation is summarized in table 3–B. Tumor was recovered from a mash prepared from submaxillary area tissue, as well as from a suspension of spleen cells from the original mouse. These tumors grew slowly, attained large sizes, and killed in 31 to 39 days.

One of the mice (table 3–B, #4) that had been inoculated with a spleen suspension was killed at 21 days after tumor inoculation, and a suspension of cells of the local tumor was inoculated into mice used in experiment S200 (table 3–C and 1). Here, too, with the inoculum taken directly from the local tumor, the tumor grew slowly, attained a large size, and killed relatively late. Treatment with 1 mg. per kg. of amethopterin failed to increase survival time (median survival time: controls, 36 days, treated, 37 days) and failed to retard the growth of the local tumor. Mice inoculated with the blood or a spleen mash of mouse #4, and not treated, showed even longer survival time and failed to "take" in a relatively high percentage of the mice. The latter observation would suggest that at the time mouse #4 was killed (21 days), unlike the sensitive L1210, the extent of infiltration had not been extensive. With the sensitive lines of L1210 at 7 or 8 days after subcutaneous inoculation of cells, transfer of blood or spleen cells invariably takes (*6*).

A second mouse of tumor generation 1 (#1) was killed on day 32 and used in experiment S201 (table 3–D). It had a large local tumor, a mash of which was inoculated into the hybrid mice (BALB/cAn × DBA/2J)F_1, the DBA/2J mice, and the BALB/cAn mice. The tumor grew progres-

derived tumors as compared with trocar implants and serial dilution of cell suspensions sitive L1210

tumor inoculation																						Tumor diameter prior to death (*average mm.*)†
24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
																						13. 0
	3	2	2		2																	11. 7 22. 2
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1		4	1	3																		27. 7
																						15. 5
												1	1		2	1	1		2	1	1	30. 6
																						12. 5
				1	3	1	2	2	1													25. 9
				1	3	1	3		1		1											25. 6
																						12. 5
																						9. 6
																						8. 6
																						8. 7
																						8. 7

‡Pooled data of expts. C40, 44, and 45. Approximately 500,000 cells in the experimental inoculum.
§Had a 9-mm. tumor prior to death.

sively in the hybrids and in the DBA/2J mice, with median survival times of 34.5 and 30.5 days, respectively. As is usual with the L1210 sensitive leukemia, it did not grow as well in the BALB/cAn mice.

Thus, the J320–3A$_7$ tumor, a variant of the L1210 sensitive tumor appearing in response to treatment with 3′,5′-dichloroamethopterin, was slow-growing and attained a large size prior to death. Since the transplant for experiment S200 was made from solid tumor mash, it would be difficult to account for the slow growth by any reduction in the number of cells of the inoculum. Also, trocar implantation of tumor behaved in the same manner (table 2). The tumor was relatively resistant to amethopterin, appeared to have the same strain specificity as the L1210 sensitive leukemia, but apparently metastasized more slowly than the sensitive line. This tumor was transferred approximately every 30 days from a spleen suspension, for 14 generations, and still grew slowly.

Tumor J325–4A$_3$

Detailed data on another slow-growing tumor variant from a mouse treated with 3′-bromo-5′-chloroamethopterin (J325–4A$_3$ tumor) is shown in table 4. The tumor appearing in the upper maxillary area and the spleen mash, which were both transplantable, grew slowly, attained large sizes, and killed late (table 4–B). Unlike the J320–3A$_7$ tumor, the J325–4A$_3$ tumor appeared to retain considerable sensitivity to amethopterin (table

TABLE 3.—Studies with the J320–3A$_7$ tumor

A. Original mouse: From expt. J320, treated daily with 28 mg. per kg. 3′,5′-dichloroamethopterin from day 8 to 60. Killed day 86. The median survival time of this group of 10 mice in expt. J320 was 32.5 days. The median survival time of controls was 11 days. In expt. J320 the leukemic cells (L1210, BB line) had been taken from spleen and inoculated subcutaneously.

Mouse #		Days following leukemic inoculation: 8	12	30	60	86	
7	Body weight (*gm.*)	26	27	27	29	22	Killed on day 86 for J320–3A$_7$, generation 1
	Tumor size (*mm.*)	10	5	0	0	0	

B. J320–3A$_7$, generation 1.

Source of inoculum	Mouse #		Days following implant (subcutaneous): 3	10	21	31	
Submaxillary node	1	Body weight (*gm.*)	26	27	29	32	Killed on day 32 for expt. S201
		Tumor size (*mm.*)	0	7	16	27	
Spleen	2	Body weight (*gm.*)	25	27	29	35	Died on day 39
		Tumor size (*mm.*)	0	0	14	25	
Spleen	3	Body weight (*gm.*)	24	26	28		Died on day 31
		Tumor size (*mm.*)	0	0	15		
Spleen	4	Body weight (*gm.*)	24	25	28		Killed on day 21 for expt. S200
		Tumor size (*mm.*)	0	6	16		
Spleen	5	Body weight (*gm.*)	24	25	27	30	Died on day 39
		Tumor size (*mm.*)	0	0	12	28	

C. Expt. S200, J320–3A$_7$, generation 2: Test for resistance of J320–3A$_7$ tumor to amethopterin. Tumor from mouse #4 of generation 1. Source of inoculum—local tumor at original site of inoculation. Route of inoculation—subcutaneous. Treatment initiated 17 days after tumor inoculation.

Source of inoculum	No. of mice	Drug	Daily dose (*mg./kg.*)	Median survival time (*days*)	Average tumor diameter (*mm.*)(*days*) 17	26	33
Local tumor suspension	9	Amethopterin	1	37	12.6	24.0	29.4
Local tumor suspension	9	Control	—	36	12.6	24.2	31.2(8)*
Blood	20	Control	—	>96 (7/20 dead on day 96)			
Spleen suspension	20	Control	—	66 (14/20 dead on day 96)			

D. Expt. S201, J320–3A$_7$, generation 2: Tumor from mouse #1 of generation 1. Source of inoculum—local tumor at original site of inoculation. Route of inoculation—subcutaneous.

No. of mice	Strain	Age (*weeks*)	Median survival time (*days*)	Average tumor diameter (*mm.*) (*days*)		
				10	20	24
6	DBA/2J	9	30. 5	9. 8	21. 6	20. 5
6	(BALB/cAn × DBA/2J)F$_1$	10	34. 5	5	16. 8(5)*	22(5)*
6	BALB/cAn	6	>90	7†	9‡	9‡

*Survivors in parentheses.

†Based on 4 of 6 mice that showed tumor growth.

‡Only 1 of 6 mice showed evidence of tumor. It subsequently regressed.

TABLE 4.—Studies with the J325–4A$_3$ tumor

A. Original mouse: Treated daily with 129 mg. per kg. 3′-bromo-5′-chloroamethopterin from day 8 to 90. On day 106 a tumor appeared in the upper maxillary area. The mouse lost weight subsequent to discontinuance of treatment and was killed on day 113. The median survival time of this group in expt. J325 was 74.5 days. The median survival time of controls was 10 days. In expt. J325 the leukemic cells (L1210, BB line) had been taken from spleen and inoculated subcutaneously.

			Days following leukemic inoculation: 8	11	30	60	90	113	
	Mouse #3	Body weight (*gm.*)	24	25	23	26	28	19	Killed on day 113 for J325–4A$_3$, generation 1
		Tumor size (*mm.*)	9	5	0	0	0	0	

B. J325–4A$_3$, generation 1

Source of inoculum	Mouse #		Days following implant (subcutaneous): 3	10	21	28	31	40	52	
Tumor (maxillary)	1	Body weight (*gm.*)	23	26	27	28	27	29	31	Dead day 59
		Tumor size (*mm.*)	0	0	5	6	6	10	30	
Tumor (maxillary)	2	Body weight (*gm.*)	26	23	23	24	25	27	30	Dead day 61
		Tumor size (*mm.*)	0	0	7	9	9	13	20	
Spleen	3	Body weight (*gm.*)	22	22	24	27	29			Dead day 39
		Tumor size (*mm.*)	0	0	11	14	15			
Spleen	4	Body weight (*gm.*)	26	26	27	30				Killed day 28. Spleen mash employed for generation 2
		Tumor size (*mm.*)	0	0	11	14				

C. J325–4A$_3$, generation 2: Spleen mash of mouse #4, generation 1 inoculated subcutaneously into stock mice. One of these was sacrificed 30 days later and the spleen mash employed for generation 3 and expt. C51.

D. Expt. C51, J325–4A$_3$, generation 3. L1210–BB sensitive generation 233: Test for resistance of J325–4A$_3$ tumor to amethopterin. Source of inoculum—spleen. Route of inoculation—subcutaneous. Treatment initiated 7 days following tumor inoculation.

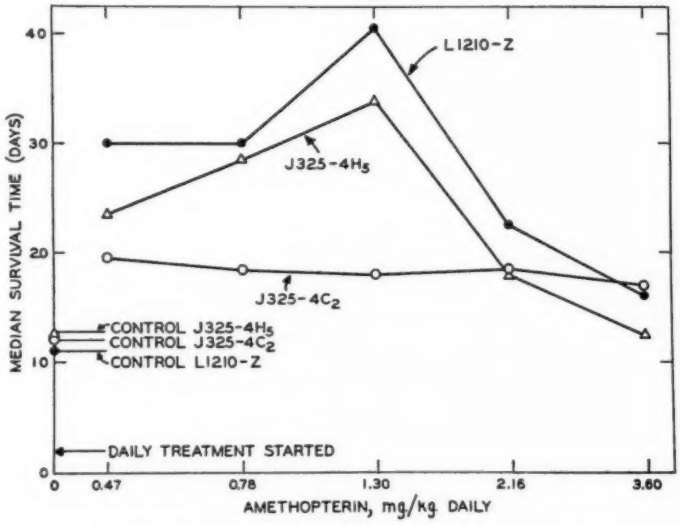
	No. of mice	Drug	Daily dose (*mg./kg.*)	Median survival time (*days*)	Average tumor diameter (*mm.*) (*days*) 7	14	23	35	50	62
J325–4A$_3$	10	Amethopterin	1	63	0	5. 4	7. 0	9. 7	15. 3(9)	20. 6(5)
	10	6-Mercaptopurine	100	24*	0	5. 1(7)	0(6)	—	—	—
	10	Controls	—	37. 5	0	6. 4	12. 0	19(7)	—	—
L1210	10	Amethopterin	1	25	11. 8	6. 3	9. 8	—	—	—
Sensitive	10	6-Mercaptopurine	100	15	12. 4	6. 9(7)	—	—	—	—
	10	Controls	—	9	12. 3	—	—	—	—	—

*6-Mercaptopurine was too toxic at the dose employed and survival time was reduced in the slow-growing J320–4A$_3$ tumor. However it did give an increase in survival time for the sensitive tumor in which fewer treatments were given. The toxicity of 6-mercaptopurine was reflected in reduction in size of local tumor and weight loss of the animals.

4–D and expt. C51). This was reflected in a marked increase in survival time of the animals treated with amethopterin, and a decrease in the rate of growth of the treated tumors. Treatment with 6-mercaptopurine inhibited the growth of the local tumor, but the dosage of drug was too high and resulted in a decrease in survival time relative to the controls.

Tumors J325–4H$_5$ and J325–4C$_2$

Text-figure 1 (expt. C40) compares the effect of amethopterin in increasing survival time of one of the sensitive L1210 lines and two fast-growing tumors derived from mice treated for an extended period with 3′-bromo-5′-chloroamethopterin. The median survival times are shown over a range of doses. The J325–4H$_5$ tumor retained its sensi-



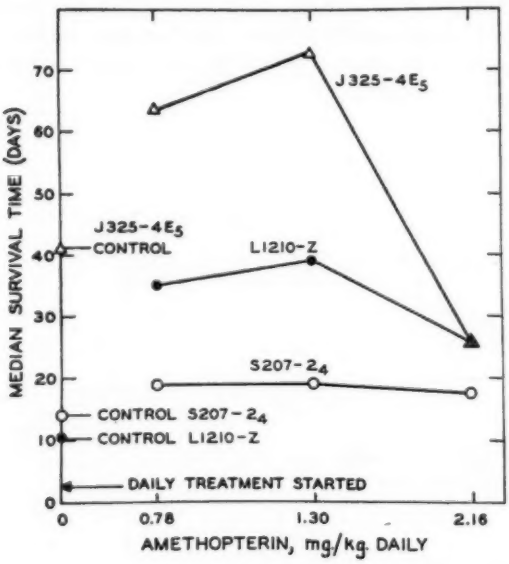
TEXT-FIGURE 1.—The effectiveness of amethopterin in increasing survival time of two tumors (J325–4H$_5$ and J325–4C$_2$) obtained from leukemic (L1210) mice treated for an extended period of time with 3′-bromo-5′-chloroamethopterin, as compared with the original sensitive leukemia (L1210–Z). Treatment was initiated 2 days after leukemic inoculation.

tivity to amethopterin, while the J325–4C$_2$ tumor was relatively resistant. The previous history of these tumors is summarized in table 1. It is of interest to note that the tumor that retained both the original rate of growth and the original sensitivity to amethopterin (J325–4H$_5$) had been treated with the lowest dose of 3′-bromo-5′-chloroamethopterin (3.6 mg./kg. daily). The J325–4C$_2$ tumor had been treated with daily doses of 46.3 mg. per kg. of 3′-bromo-5′-chloroamethopterin.

*6-Mercaptopurine was too toxic at the dose employed and survival time was reduced in the slow-growing J320–4As tumor. However it did give an increase in survival time for the sensitive tumor in which fewer treatments were given. The toxicity of 6-mercaptopurine was reflected in reduction in size of local tumor and weight loss of the animals.

Tumors S207–2_4 and J325–$4E_5$

Text-figure 2 (expt. C44) shows the median survival time, over a range of doses, for a fast-growing, relatively resistant variant, S207–2_4, (table 1) derived after treatment with 75 mg. per kg. daily of 3′,5′-dichloroamethopterin. The control median survival time of 14 days was increased, at optimal doses, to 19 days. In contrast, the median survival time for the L1210–Z leukemia was increased from 10 days to 39 days in the controls. A second tumor, J325–$4E_5$, (table 1) obtained from a mouse treated with 16.7 mg. per kg. 3′-bromo-5′-chloroamethopterin grew

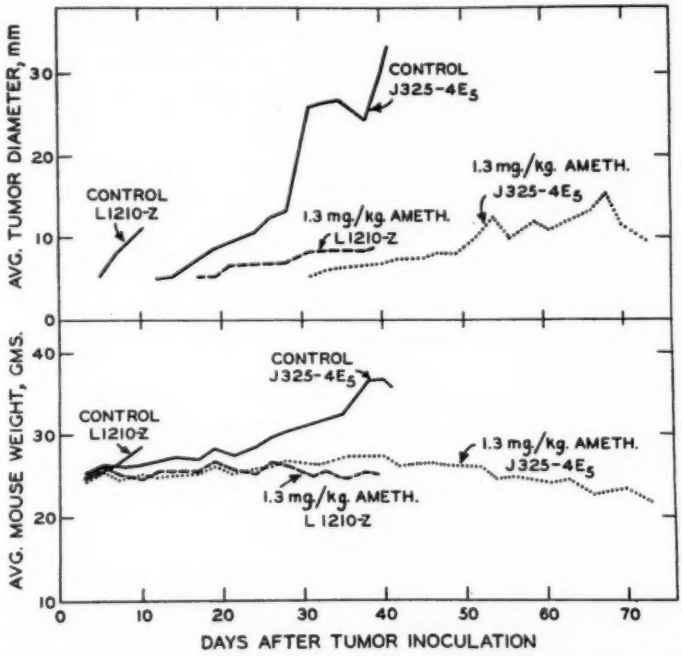


TEXT-FIGURE 2.—The effectiveness of amethopterin in increasing the survival time of two tumors (J325–$4E_5$ and S207–2_4) obtained from leukemic (L1210) mice treated for an extended period of time respectively with 3′-bromo-5′-chloroamethopterin and 3′,5′-dichloroamethopterin, as compared with the sensitive leukemia (L1210–Z). Treatment was initiated 3 days after leukemic inoculation.

slowly and had a median survival time of 41 days for controls. Although the increase in survival time elicited by amethopterin with the J325–$4E_5$ tumor was not extensive, relative to controls, there was nevertheless considerable sensitivity that was reflected in a 32-day increase in median survival time on treatment with amethopterin (median survival time, 73 days). With the J325–$4E_5$ tumor, the experimental inoculum was 100 times greater than that giving 100 percent death in serial dilution of the inoculum, which indicated that the long survival time of controls was not attributable to any reduction in potency of the inoculum. Extensive survival time was also observed with trocar implantation of this

tumor (table 2). At one one-hundreth of the experimental inoculum of the L1210–Z tumor, the animals were all dead by the 12th day.

Text-figure 3 (expt. C44) compares tumor growth and body-weight changes for the J325–4E$_5$ tumor and the L1210–Z leukemia, with and without treatment by amethopterin. In the J325–4E$_5$ controls the local tumor at the site of inoculation appeared later than in the L1210–Z, and achieved a considerably larger size prior to death. On treatment with the optimal dose level of amethopterin (1.3 mg./kg.; *see* text-fig. 2), tumor growth was retarded for both tumors. Although the local tumors



TEXT-FIGURE 3.—Comparison of tumor diameter and mouse weight of amethopterin-treated and control mice for the variant J325–4E$_5$ leukemia and the sensitive L1210–Z leukemia (*see* text-fig. 2). Treatment was initiated 3 days after leukemia inoculation.

appeared later in the J325–4E$_5$ treated mice, the tumors eventually achieved larger sizes than in the L1210–Z treated group.

Total-body weight of mice with the L1210–Z line showed little change in the short time to death (text-fig. 3). The marked increase in total-body weight for the mice with the J325–4E$_5$ tumor would appear to be a reflection of the increased growth of the tumor. The relatively equal and stable body weights, on treatment with amethopterin, are indicative of equivalent and fairly extensive host tolerance to the optimal dosage of drug, for mice with the original and variant tumor.

Tumors J325–4C_9, J325–4A_2, and S207–2_5

Data on 3 additional tumors derived from long-term survivors are summarized in table 5 (expt. C45 and table 1). The J325–4C_9 tumor derived from a mouse treated with 46.3 mg. per kg. 3′-bromo-5′-chloroamethopterin was slow-growing and showed a 27-day increase in median survival time on treatment with amethopterin. The increase in survival time relative to controls was low, however, since there was a long survival time for the controls (median survival time, 66.5 days). With this tumor only one of three of the tumor-titration group died when 0.1 ml. of the experimental inoculum was given, which indicates that at least part of the slow growth of the tumor resulted from a reduced number of leukemic cells in the inoculum. That this tumor is slow-growing, however, is substantiated by the extensive survival time obtained on implantation of solid tumor (table 2). If the concentration of the inoculum had indeed been low, this would, in turn, suggest that the tumor is quite resistant. At reduced inoculum levels with the sensitive L1210 tumor, extensive survival time and "no takes" would be expected, particularly when treatment with amethopterin is initiated early (*11*, *12*).

TABLE 5.—Influence of amethopterin on survival time of mice inoculated with the sensitive L1210–Z and three sublines*

Tumor	Amethopterin, daily dose (*mg./kg.*)			
	0	0. 77	1. 29	2. 16
	Median survival time (*days*)			
J325–4C_9	66. 5	86	93. 5	24. 5
J325–4A_2	10	31	34	18. 5
S207–2_5	9. 5	15	17. 5	16
L1210–Z	13	40	64. 5	21

*Treatment initiated on day 2. *See* table 1 and expt. C45.

The J325–4A_2 tumor derived from a mouse treated with 129 mg. per kg. of 3′-bromo-5′-chloroamethopterin was fast-growing and quite sensitive, and showed a 3.4-fold increase in median survival time on treatment with amethopterin (tables 1 and 5).

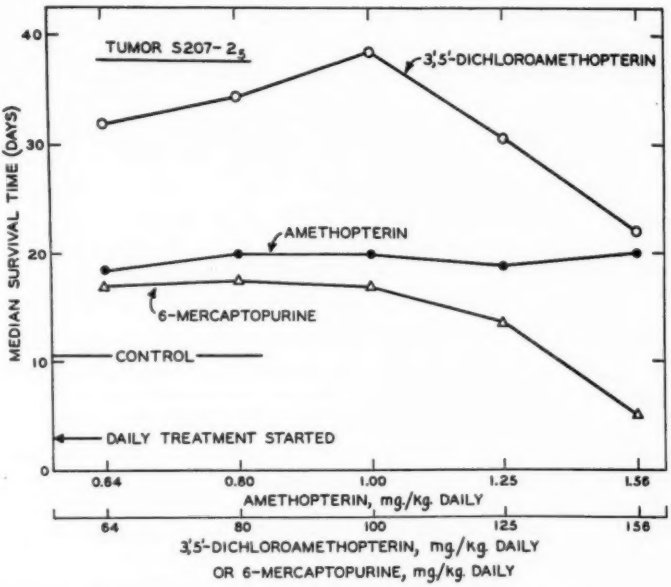
The S207–2_5 tumor derived from a mouse treated with 75 mg. per kg. of dichloroamethopterin continued to grow rapidly, and showed partial resistance to amethopterin (tables 1 and 5).

In experiment C45 (table 5) the L1210–Z tumor showed greater sensitivity than is ordinarily observed. The titration of the L1210–Z experimental inoculum in this experiment indicated that it was not as potent as usual. The animals succumbed at 0.1 but not at 0.01 ml. of the experimental inoculum.

Relative Response of a 3′,5′-Dichloroamethopterin-Resistant Variant (S207–2_5) of L1210 to 3′,5′-Dichloroamethopterin and Amethopterin

Since the S207–2_5 tumor derived from a mouse treated with 3′,5′-dichloroamethopterin showed partial cross-resistance to amethopterin, an

experiment was conducted to determine whether it would have the same or an altered degree of resistance to 3′,5′-dichloroamethopterin (text-fig. 4, table 1, and expt. C46). The sensitivity of the tumor to treatment with 6-mercaptopurine was also tested. The data indicate that the S207–2_5 tumor was less resistant to treatment with 3′,5′-dichloroamethopterin than to amethopterin. Optimal treatment with amethopterin in this experiment elicited a 1.9-fold increase in median survival time over controls, while treatment with 3′,5′-dichloroamethopterin increased



TEXT-FIGURE 4.—Comparison of the relative effectiveness of 3′,5′-dichloroamethopterin, amethopterin, and 6-mercaptopurine in increasing survival time of the S207–2_5 tumor variant obtained from a leukemic (L1210) mouse treated with 3′,5′-dichloroamethopterin.

median survival time 3.7-fold. However, the increase in survival time with 3′,5′-dichloroamethopterin was considerably less than that usually observed with the L1210 sensitive leukemia (*2–5*). Thus, 3′,5′-dichloroamethopterin, which is more effective than amethopterin in increasing survival time of the sensitive line of L1210, was also more effective than amethopterin against a somewhat resistant line.

Treatment with 6-mercaptopurine was almost as effective as treatment with amethopterin in increasing the survival time of mice with the S207–2_5 tumor. Against the sensitive L1210, 6-mercaptopurine is ordinarily about 50 to 60 percent as effective as amethopterin (*6, 8*).

Relative Response of an Amethopterin-Resistant Variant of L1210 (M46R) to 3′,5′-Dichloroamethopterin and Amethopterin

Since a resistant variant of leukemia L1210 arising in response to treatment with 3′,5′-dichloroamethopterin was more resistant to treatment with amethopterin than to 3′,5′-dichloroamethopterin, it was considered of interest to study the reverse situation in which a resistant variant of L1210 (M46R) had appeared in response to therapy with amethopterin.

The results of an experiment comparing the effectiveness of amethopterin, 3′,5′-dichloroamethopterin, and 6-mercaptopurine in increasing the lifespan of mice with this leukemia (M46R) are summarized in table 6. The tumor virtually was completely resistant to treatment with amethopterin. In contrast, 3′,5′-dichloroamethopterin was still capable of giving a 5.5-day increase in survival time over controls. 6-Mercaptopurine was somewhat more effective than the dichlorinated compound giving a 7.5-day increase in survival time. The greater therapeutic effectiveness against the sensitive leukemia L1210 of both 3′,5′-dichloroamethopterin (median survival time, 64.5 days at a dose of 98 mg./kg. daily) and amethopterin (median survival time, 29 days at a dose of 0.98 mg./kg. daily) may also be noted in table 6. The resistant tumor and sensitive L1210 had approximately the same degree of responsiveness to treatment with 6-mercaptopurine.

TABLE 6.—Relative effectiveness of 3′,5′-dichloroamethopterin, amethopterin, and 6-mercaptopurine in increasing survival time of mice with the sensitive L1210 and an amethopterin-resistant subline*

	Compound	Optimal daily dose (*mg./kg.*)	Median survival time (*days*)
M46R	3′,5′-Dichloroamethopterin	140	16
	6-Mercaptopurine	140	18
	Amethopterin	0. 48–2. 0	11
	Controls	—	10. 5
		Daily dose	
L1210–Z	3′,5′-Dichloroamethopterin	98	64. 5
	6-Mercaptopurine	98	18
	Amethopterin	0. 90	29
	Controls	—	12. 0

*Cells per mouse for each tumor, 400,000; treatment day, 8 to 60; L1210–Z—Generation 224; M46R—Generation 60; expt. S242.

DISCUSSION

Marshall (*13*) emphasized that in infection-chemotherapy, consideration should be given to the triad of the host, parasite, and drug. Similarly, in antitumor chemotherapy the host-tumor-drug relationship governs drug effectiveness (*11, 14–16*).

Two of the factors that may alter the host-tumor-drug relationship and thereby limit the employment of the available antineoplastic agents are

the toxicity of the drugs for the host and the development of resistance to drug therapy.

Most of the known antineoplastic agents are toxic for the host as well as for the tumor. So long as the available drugs showed relatively little selective, antitumor action, the toxicity of the drugs for the host *per se* could reasonably account for the failure of therapy after a relatively short period of treatment. In the management of mouse leukemia (L1210) with antagonists of folic acid, tolerated dosages of drug increased the survival time, but permitted progressive growth of the tumor (*12*). Higher dosages caused more extensive retardation of tumor growth, but the animals succumbed primarily to drug toxicity.

The selection or appearance of resistant, variant cells also could result in reduced drug effectiveness. Burchenal *et al.* demonstrated the emergence of resistant cells in response to amethopterin treatment of leukemic mice (*17*). The phenomenon has now been investigated in various systems (*18–26*). As has been pointed out by Skipper *et al.* (*27*), since most of the available antineoplastic drugs have a low order of antitumor specificity of action, even a small change in the degree of tumor-cell resistance could result in the drug becoming ineffective.

In previous studies with leukemia L1210 in which treatment with amethopterin was initiated early in the course of the disease, extensive survival times and a low but significant number of total recoveries from the leukemic implant were observed (*12*). Also, the leukemic cells of several of the animals that had survived for a relatively extended period of time showed little or no evidence of resistance (*12*). Although these observations did not rule out the possibility that the origin of a low level of resistance may have interfered with therapy, they did suggest that, with this leukemia, the problem of the appearance of a large population of resistant variants did not necessarily enter as a major factor in the failure of therapy.

Nevertheless, with the advent of compounds with greater antileukemic action, such as the ones employed in the current study, the toxicity of the drugs would appear to diminish in importance as a limiting factor, while other factors such as the origin of resistant variants may become increasingly important as limitations to therapy. Various possible mechanisms leading to drug ineffectiveness, such as the augmentation of enzymatic detoxification mechanisms, increase in biosynthesis or concentration of a metabolite, changes in cell permeability, and the development of alternate pathways, are discussed by Law (*20*). In the current series of experiments (*5*), the halogenated derivatives of amethopterin were capable of increasing the survival time for an extended period, and some of the animals were apparently cured of the disease, even when the treatment was started after the disease was systemic. Nevertheless, the ultimate failure of treatment, when it occurred, may, at least in some cases, be attributable to the origin of resistant, variant tumor cells.

The current studies show that with extensive therapy a whole spectrum of variants may arise, which show altered rates of growth, apparently

altered degrees of invasiveness, and a wide range of sensitivity to the drugs, including highly sensitive as well as almost completely resistant forms. Some of the tumors derived after extensive therapy retained their original rate of growth, and killed in a short time like the original sensitive leukemia (L1210). Others grew slowly at the site of leukemic inoculation, achieved large sizes prior to death, and gave very extensive survival times. There did not seem to be any definite correlation between the rate of growth of the tumor and the degree of resistance. Both rapid-growing and slow-growing tumors demonstrated varying degrees of resistance. It is noteworthy that altered forms of tumor-cell populations appearing at various sites have been observed with a plasma-cell neoplasm (*21*, *24*). Treatment of the plasma-cell neoplasm with DON (6-diazo-5-oxo-L-norleucine) and with azaserine (O-diazoacetyl-L-serine) resulted in the appearance of resistant variants, in some instances in a single generation. The altered forms observed in the current study may resemble the "late recurrences" which were noted with the plasma-cell neoplasm on treatment with the glutamine antagonists.

The variants appeared to be relatively stable in serial passage, without further treatment. One of the slow-growing tumors that attained large sizes and showed long survival times retained these characteristics after 15 generations (table 2).

The S207–2_8 tumor had not lost any of its acquired resistance after 6 generations (table 1). After 16 generations, however, it was only slightly more resistant to amethopterin than the parent sensitive line, and no difference could be demonstrated with 3′,5′-dichloroamethopterin.

It is of interest that resistant tumor variants remained more responsive to treatment with dichloroamethopterin than with amethopterin, whether they arose from therapy with 3′,5′-dichloroamethopterin itself or with amethopterin. This would appear to be a reflection of the more extensive therapeutic effectiveness of the halogenated derivative, as demonstrated with the sensitive leukemia. That there was a reduction in effectiveness, however, for both drugs, as compared with the sensitive leukemia, suggests that the drugs exert their therapeutic action via similar mechanisms. This interpretation is supported by the observation that the antileukemic action of both 3′,5′-dichloroamethopterin and amethopterin may be reversed by citrovorum factor (*5*). The foregoing observations would suggest that the appearance of resistant variants may be combated by obtaining more effective structural analogues acting at the same sites, as well as by employing agents with different sites of action.

In agreement with the previous observations of Law (*20*), 6-mercaptopurine retained its effectiveness, and there was no increase in collateral sensitivity against the resistant variants.

To what extent the slow-growing tumors and the resistant variants obtained here were the result of random mutation and selection, as was demonstrated for amethopterin with this leukemia (*28*), has not been clarified. That the original inoculum of sensitive L1210 did not contain any sizable proportion of resistors is suggested by (*a*) the recovery of some

tumor lines that remained sensitive and (*b*) the apparent total eradication of tumor in some of the mice. It has been observed (*5*) that mice apparently cured of systemic leukemia L1210 by treatment with halogenated derivatives of amethopterin are resistant to reimplantation of the tumor. Reinoculation of leukemic cells either failed to take or a local tumor appeared, which grew slowly and achieved a large size prior to death. This observation raises the question of the relative contributions of direct antimetabolite effect by the drug, and the elicitation of immune response to the total antileukemic action. The unmasking of immunological differences on extensive therapy, or drug-induced alterations in host-tumor compatability relationships conceivably may have contributed not only to the resistance to reinoculation but also to the origin of the slow-growing and resistant variants. The importance of immunological mechanisms in influencing tumor growth has been amply demonstrated by numerous investigators (*29–32*).

That the recovered tumors stem from the original leukemic inoculum is indicated by the similarity in histological appearance. The possibility of carcinogenic action of the halogenated derivatives was tested. Twenty nonleukemic mice were treated daily with 75 mg. per kg. of dichloroamethopterin for 90 days and then observed for another 60 days. There was no evidence of appearance of tumor in any of these mice.

Whether the appearance of the variant tumors may be related to a possible association of a transmissible tumor agent (*33*) has not been determined.

The ability to obtain very extensive survival time with the halogenated derivatives of amethopterin may facilitate study of the origin and nature of tumor variants and provide additional tumor material for investigation of the phenomenon of resistance in chemotherapy.

REFERENCES

(*1*) Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A.: Temporary remissions in acute leukemia in children produced by folic acid antagonists, 4-aminopteroyl glutamic acid (aminopterin). New England J. Med. 238: 787–793, 1948.

(*2*) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: Comparison of the relative effectiveness of folic acid congeners against advanced leukemia in mice. J. Nat. Cancer Inst. 19: 1133–1135, 1957.

(*3*) Goldin, A., Venditti, J. M., Humphreys, S. R., Shuster, L., Darrow, R. A., and Mantel, N.: Antileukemic action of folic acid analogs. Abstracts of papers 133rd meeting, Am. Chem. Soc. 25–26 M, San Francisco, Calif., Apr. 13–18, 1958.

(*4*) ———: Advanced leukemia as a tool for chemotherapeutic studies. Acta Unio internat contra cancrum. In press.

(*5*) Goldin, A., Humphreys, S. R., Venditti, J. M., and Mantel, N.: Prolongation of the lifespan of mice with advanced leukemia (L1210) by treatment with halogenated derivatives of amethopterin. J. Nat. Cancer Inst. 22: 811–823, 1959.

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(6) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: Quantitative evaluation of chemotherapeutic agents against advanced leukemia in mice. J. Nat. Cancer Inst. 21: 495–511, 1958.

(7) ———: Modification of treatment schedules in the management of advanced mouse leukemia with amethopterin. J. Nat. Cancer Inst. 17: 203–212, 1956.

(8) Venditti, J. M., Humphreys, S. R., Mantel, N., and Goldin, A.: Combined treatment of advanced leukemia (L1210) in mice with amethopterin and 6-mercaptopurine. J. Nat. Cancer Inst. 17: 631–638, 1956.

(9) Humphreys, S. R., and Goldin, A. Unpublished observations.

(10) Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(11) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: The influence of the concentration of leukemic inoculum on the effectiveness of treatment. Science 123: 840, 1956.

(12) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., and Mantel, N.: Studies on the management of mouse leukemia (L1210) with antagonists of folic acid. Cancer Res. 15: 742–747, 1955.

(13) Marshall, E. K., Jr.: The dosage schedule of chemotherapeutic agents. Pharmacol. Rev. 4: 85–105, 1952.

(14) Goldin, A., Mantel, N., Greenhouse, S. W., Venditti, J. M., and Humphreys, S. R.: Factors influencing the specificity of action of an antileukemic agent (aminopterin). Time of treatment and dosage schedule. Cancer Res. 14: 311–314, 1954.

(15) ———: Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of aminopterin in mice. Cancer Res. 14: 43–48, 1954.

(16) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., and Mantel, N.: Factors influencing the specificity of action of an antileukemic agent (aminopterin): Host age and weight. J. Nat. Cancer Inst. 16: 709–721, 1955.

(17) Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N.: The induction of resistance to 4-amino-N^{10}-methylpteroyl glutamic acid in a strain of transmitted mouse leukemia. Science 111: 116–117, 1950.

(18) Law, L. W., and Boyle, P. J.: Development of resistance to folic acid antagonists in a transplantable lymphoid leukemia. Proc. Soc. Exper. Biol. & Med. 74: 599–602, 1950.

(19) Law, L. W.: Observations on properties of leukemic cells resistant to folic acid antagonists. J. Nat. Cancer Inst. 11: 849–865, 1951.

(20) ———: Recent advances in experimental leukemia research. Cancer Res. 14: 695–709, 1954.

(21) Potter, M., and Law, L. W.: Studies of a plasma-cell neoplasm of the mouse. I. Characterization of neoplasm 70429, including its sensitivity to various antimetabolites with rapid development of resistance to azaserine, DON, and *N*-methylformamide. J. Nat. Cancer Inst. 18: 413–441, 1957.

(22) Burchenal, J. H., and Holmberg, E. A. D.: The utility of resistant leukemias in screening for chemotherapeutic activity. Ann. New York Acad. Sc. 76: 826–831, 1958.

(23) Law, L. W.: Some aspects of drug resistance in neoplasms. Ann. New York Acad. Sc. 71: 976–993, 1958.

(24) Potter, M.: Variation in resistance patterns in different neoplasms. Ann. New York Acad. Sc. 76: 630–642, 1958.

(25) Klein, G., and Klein, E.: The evolution of independence from specific growth stimulation and inhibition in mammalian tumour-cell populations. Symposia Soc. Exper. Biol. No. 11. The Biological Action of Growth Substances 306–328, 1957.

(*26*) NICHOL, C. A., and WELCH, A. D.: Metabolic requirements for formation of citrovorum factor and studies of mechanism of resistance to A-methopterin. *In* Antimetabolites and Cancer (Rhoads, C. P., ed.). AAAS Publication, 1955, pp. 63–87.

(*27*) SKIPPER, H. E., SCHABEL, F. M., JR., BELL, M., THOMSON, J. R., and JOHNSON, S.: On the curability of experimental neoplasms. I. A-methopterin and mouse leukemias. Cancer Res. 17: 717–726, 1957.

(*28*) LAW, L. W.: The phenomena of resistance and dependence in leukemic cells. *In* Ciba Foundation Symposium on Leukemia Research (Wolstenholme, G. E. W., and Cameron, M. P., eds.). London, J. & A. Churchill, Ltd. vol. 14, 1954, pp. 105–129.

(*29*) KALISS, N., and BRYANT, B. F.: Factors determining homograft destruction and immunological enhancement in mice receiving successive tumor inocula. J. Nat. Cancer Inst. 20: 691–704, 1958.

(*30*) SNELL, G. D.: Histocompatability genes of the mouse. I. Demonstration of weak histocompatability differences by immunization and controlled tumor dosage. J. Nat. Cancer Inst. 20: 787–824, 1958.

(*31*) BARRETT, M. K., and HANSEN, W. H.: Resistance of mice to multiple inoculations of a transplantable tumor J. Nat. Cancer Inst. 15: 411–420, 1954.

(*32*) PREHN, R. T., and MAIN, J. M.: Immunity to methylcholanthrene-induced sarcomas. J. Nat. Cancer Inst. 18: 769–778, 1957.

(*33*) STEWART, S. E., EDDY, B. E., and BORGESE, N.: Neoplasms in mice inoculated with a tumor agent carried in tissue culture. J. Nat. Cancer Inst. 20: 1223–1243, 1958.

(6) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: Quantitative evaluation of chemotherapeutic agents against advanced leukemia in mice. J. Nat. Cancer Inst. 21: 495–511, 1958.

(7) ———: Modification of treatment schedules in the management of advanced mouse leukemia with amethopterin. J. Nat. Cancer Inst. 17: 203–212, 1956.

(8) Venditti, J. M., Humphreys, S. R., Mantel, N., and Goldin, A.: Combined treatment of advanced leukemia (L1210) in mice with amethopterin and 6-mercaptopurine. J. Nat. Cancer Inst. 17: 631–638, 1956.

(9) Humphreys, S. R., and Goldin, A. Unpublished observations.

(10) Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(11) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: The influence of the concentration of leukemic inoculum on the effectiveness of treatment. Science 123: 840, 1956.

(12) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., and Mantel, N.: Studies on the management of mouse leukemia (L1210) with antagonists of folic acid. Cancer Res. 15: 742–747, 1955.

(13) Marshall, E. K., Jr.: The dosage schedule of chemotherapeutic agents. Pharmacol. Rev. 4: 85–105, 1952.

(14) Goldin, A., Mantel, N., Greenhouse, S. W., Venditti, J. M., and Humphreys, S. R.: Factors influencing the specificity of action of an antileukemic agent (aminopterin). Time of treatment and dosage schedule. Cancer Res. 14: 311–314, 1954.

(15) ———: Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of aminopterin in mice. Cancer Res. 14: 43–48, 1954.

(16) Goldin, A., Venditti, J. M., Humphreys, S. R., Dennis, D., and Mantel, N.: Factors influencing the specificity of action of an antileukemic agent (aminopterin): Host age and weight. J. Nat. Cancer Inst. 16: 709–721, 1955.

(17) Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N.: The induction of resistance to 4-amino-N^{10}-methylpteroyl glutamic acid in a strain of transmitted mouse leukemia. Science 111: 116–117, 1950.

(18) Law, L. W., and Boyle, P. J.: Development of resistance to folic acid antagonists in a transplantable lymphoid leukemia. Proc. Soc. Exper. Biol. & Med. 74: 599–602, 1950.

(19) Law, L. W.: Observations on properties of leukemic cells resistant to folic acid antagonists. J. Nat. Cancer Inst. 11: 849–865, 1951.

(20) ———: Recent advances in experimental leukemia research. Cancer Res. 14: 695–709, 1954.

(21) Potter, M., and Law, L. W.: Studies of a plasma-cell neoplasm of the mouse. I. Characterization of neoplasm 70429, including its sensitivity to various antimetabolites with rapid development of resistance to azaserine, DON, and *N*-methylformamide. J. Nat. Cancer Inst. 18: 413–441, 1957.

(22) Burchenal, J. H., and Holmberg, E. A. D.: The utility of resistant leukemias in screening for chemotherapeutic activity. Ann. New York Acad. Sc. 76: 826–831, 1958.

(23) Law, L. W.: Some aspects of drug resistance in neoplasms. Ann. New York Acad. Sc. 71: 976–993, 1958.

(24) Potter, M.: Variation in resistance patterns in different neoplasms. Ann. New York Acad. Sc. 76: 630–642, 1958.

(25) Klein, G., and Klein, E.: The evolution of independence from specific growth stimulation and inhibition in mammalian tumour-cell populations. Symposia Soc. Exper. Biol. No. 11. The Biological Action of Growth Substances 306–328, 1957.

(*26*) NICHOL, C. A., and WELCH, A. D.: Metabolic requirements for formation of citrovorum factor and studies of mechanism of resistance to A-methopterin. *In* Antimetabolites and Cancer (Rhoads, C. P., ed.). AAAS Publication, 1955, pp. 63–87.

(*27*) SKIPPER, H. E., SCHABEL, F. M., JR., BELL, M., THOMSON, J. R., and JOHNSON, S.: On the curability of experimental neoplasms. I. A-methopterin and mouse leukemias. Cancer Res. 17: 717–726, 1957.

(*28*) LAW, L. W.: The phenomena of resistance and dependence in leukemic cells. *In* Ciba Foundation Symposium on Leukemia Research (Wolstenholme, G. E. W., and Cameron, M. P., eds.). London, J. & A. Churchill, Ltd. vol. 14, 1954, pp. 105–129.

(*29*) KALISS, N., and BRYANT, B. F.: Factors determining homograft destruction and immunological enhancement in mice receiving successive tumor inocula. J. Nat. Cancer Inst. 20: 691–704, 1958.

(*30*) SNELL, G. D.: Histocompatability genes of the mouse. I. Demonstration of weak histocompatability differences by immunization and controlled tumor dosage. J. Nat. Cancer Inst. 20: 787–824, 1958.

(*31*) BARRETT, M. K., and HANSEN, W. H.: Resistance of mice to multiple inoculations of a transplantable tumor J. Nat. Cancer Inst. 15: 411–420, 1954.

(*32*) PREHN, R. T., and MAIN, J. M.: Immunity to methylcholanthrene-induced sarcomas. J. Nat. Cancer Inst. 18: 769–778, 1957.

(*33*) STEWART, S. E., EDDY, B. E., and BORGESE, N.: Neoplasms in mice inoculated with a tumor agent carried in tissue culture. J. Nat. Cancer Inst. 20: 1223–1243, 1958.

Effect of Trypsin on Growth in Several Clones of Tissue Cells[1, 2]

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SUMMARY

The effects of trypsin (Armour's Tryptar) on three cell strains—NCTC clone 929 (strain L), NCTC clone 1469 (mouse liver), and HeLa—were studied. Concentrations of 125 mg. percent and above destroyed the cells of all three strains cultivated in biological medium. In strain L and HeLa, there was a decided increase in cell number in the trypsin media (62.5, 31, and 16 mg. % trypsin) as compared to the controls, but trypsin had very little, if any, effect on mouse liver cells. Horse serum "predigested" with trypsin (62.5 mg. % and lower) before addition to the medium had an effect similar to whole medium exposed to trypsin—a pronounced increase in cell number, whereas liver-cell growth was retarded. Embryo extract "predigested" with trypsin had effects on strain L and liver cells similar to that of the "predigested" horse serum; however, it had no effect on HeLa cells. Trypsin, 62.5 mg. percent and above, was toxic to strain L cells cultivated in a semisynthetic medium, while 31 mg. percent retarded growth. Very little effect was noted on the cells exposed to 16 or 10 mg. percent trypsin in this medium, although there may have been a slight stimulation in the 10 mg. percent trypsin. Indications are that the beneficial effects of trypsin are due chiefly to the action of the enzyme on the medium while the toxic effects are caused by its direct action on the cells.—J. Nat. Cancer Inst. 23: 655–667, 1959.

ONE OF the earliest records of the utilization of trypsin for preparation of suspensions of living cells was by Rous and Jones (*1*). This has led to various uses of trypsin, such as in the preparation of monkey kidney cells for poliomyelitis studies, reported by Youngner (*2*). Since that time many investigators have used the method. In recent years, trypsin has been employed widely in the transplantation of cell cultures. Rinaldini (*3*) presented a very helpful review, on the isolation of living cells from tissues, in which he discussed the use of trypsin and other enzymes. Moscona (*4*, *5*) and Moscona and Moscona (*6*) succeeded in breaking up embryonic rudiments into suspensions of viable cells by digestion of the

[1] Received for publication March 9, 1959.

[2] This investigation was supported by research grant C-1654 from the National Cancer Institute, National Institutes of Health, Public Health Service, and in part by funds received from the Cooper Foundation, Lincoln, Nebraska, through the University of Nebraska Foundation.

intercellular matrix in a dilute trypsin solution. Frisch and Jentoft (*7*) used it in the preparation of monkey testicular cells, and Chang (*8*) used it for preparation of epithelium-like cells from a variety of human tissues.

However, there has always been some question as to whether or not trypsin has a beneficial effect on the separated cells. Simms and Stillman (*9, 10*) have shown that certain concentrations of trypsin will accelerate the growth of adult tissue *in vitro*. They suggest that this stimulating effect may be due to proteolytic digestion of the tissue substance and the apparent removal of an inhibitor contained in it.

Fermi (*11*) demonstrated that trypsin, even in relatively high concentrations, was not detrimental to living cells. More recently, others have confirmed this: Medawar (*12*); Runnstrom, Monne, and Broman (*13*); Northrop (*14*); and Meyer and Rapport (*15*). On the contrary, the work of Levan and Biesele (*16*) and Magee, Sheek, and Sagik (*17*) suggested that trypsinization caused damage to cells. Northrop (*18*), in ascertaining the permeability of living and dead cells to trypsin, found that as long as the cells were living no detectable quantity of enzyme was taken up by them; however, in the presence of dead cells, the enzyme disappeared rapidly from the solution and became concentrated within these cells. This was attributed to the increase in cell permeability in dead cells. Thus the advantage of using trypsin to digest the cell matrix as a means for establishing cell suspensions rests in the fact that there seems to be no danger, insofar as injury to the living cell is concerned, as long as the trypsin concentration is not too great. Nevertheless, although trypsin, as used by the investigators mentioned previously, did not appear to affect living cells, it is possible that prolonged contact may have an over-all effect on the physiological constitution of the cell.

The present study was undertaken in an attempt to ascertain the effect or effects of various concentrations of trypsin on growth of clones of several types of tissue cells cultivated *in vitro*.

MATERIALS AND METHODS

In these investigations we used a highly purified crystalline trypsin prepared by the Armour Laboratories under the commercial name Tryptar, which contains 95 to 98 percent trypsin. In dry form, Tryptar is stable at room temperature. For use, it was dissolved in Sorensen's phosphate buffer solution, *p*H 7.1, prepared by dissolving 6.6 mg. per ml. of anhydrous Na_2HPO_4 and 2.7 mg. per ml. of anhydrous KH_2PO_4 in distilled water. This solution will retain 80 percent of its proteolytic activity up to 72 hours at room temperature.

Strain L cells (NCTC clone 929), mouse liver epithelium (NCTC clone 1469), and HeLa cells were selected for these experiments. Replicate cultures were prepared in Carrel D-3.5 flasks.

The cells were cultivated in a medium consisting of 40 percent Earle's balanced salt solution, 40 percent horse serum, and 20 percent chick-embryo extract (EE:50). The H-ion concentration was adjusted to *p*H 7.4.

Stock cultures were maintained in T-60 flasks and, when needed, the cells were scraped from the glass surface by means of a microrake or platinum-wire loop. A uniform suspension was established by drawing the cells in and out of a sterile pipette, which separated most of the cells. The desired quantity of fresh medium was added and the suspension placed in the reservoir of the replicator, as described by Pace and Aftonomos (*19*).

For each experiment, 10 Carrel flasks were used for control cultures and 10 for each of the different trypsin concentrations studied. To each flask was added 1 ml. of the cell suspension in the desired medium, by means of the replicator. The trypsin-treated cultures were subdivided into 5 groups; they contained the same medium as the controls except that trypsin was added in the following concentrations: 250, 125, 62.5, 31, and 16 mg. percent. All the cultures were placed in an incubator at 37.5° C. for 48 hours, at which time the medium in each flask was replaced with fresh medium. At the same time 2 flasks were selected at random from the control group and 2 from each concentration of the experimental group so that counts could be made. For this purpose, an 0.9 percent NaCl solution containing 3 percent trypsin was used to free the cells of the strain L and HeLa cultures.

However, this concentration of trypsin did not free the liver cells satisfactorily. Therefore, a solution of 0.1 M citric acid with crystal violet was used for nuclei enumeration of the liver cells. The method of counting was similar to that used by Sanford *et al.* (*20*).

Since it is now evident that initial cell numbers have an influence at least on the rapidity at which cultures reach their maximum cell numbers [Earle *et al.* (*21*) and Pace and Aftonomos (*19*)], this was considered in the following analysis. The total number of cultures studied for each initial cell-density range as well as for each trypsin concentration was noted.

RESULTS

Effect of Trypsin on Cells Cultivated in Serum and Embryo-Extract Medium

NCTC clone 929 (strain L).—Strain L cells are used widely for physiological studies and they were used initially in this study.

It was found that the cells exposed to 250 and 125 mg. percent trypsin failed to survive. They detached in the higher concentration and for the most part disintegrated by the 2d day. Although the cells in 125 mg. percent trypsin actually proliferated to some extent during the first 48 hours, they died by the 3d day.

Initial cell inoculums differed in the various experiments as shown in table 1. The results of the effects of different trypsin concentrations on

TABLE 1.—Effects of different concentrations of trypsin on growth of three strains of tissue cells (strain L, mouse liver, and HeLa) cultivated *in vitro* with different initial cell numbers*

Cell type	Experimental group	Age of cultures (*days*)	Concentration of trypsin (*mg. %*)			
			0 (Control)	16	31	62.5
			Total number of cells (*millions per ml.*)			
NCTC clone 929 (Earle's strain L)	A (50 cultures, 5 expts.)	0	0. 45	0. 45	0. 45	0. 45
		2	1. 06	2. 45	2. 78	2. 77
		4	2. 76	5. 49	5. 27	5. 92
		6	3. 75	7. 10	7. 16	8. 22
		8†	3. 31	8. 02	9. 41	9. 26
		10†	3. 68			
	B (30 cultures, 3 expts.)	0	0. 65	0. 65	0. 65	0. 65
		2	0. 81	0. 81	0. 93	1. 02
		4	0. 93	1. 14	1. 42	2. 11
		6	1. 40	1. 79	2. 24	4. 14
		8	2. 26	3. 19	5. 26	7. 72
		10	2. 77	6. 62	8. 22	
		12	3. 25	9. 25		
NCTC clone 1469 (mouse liver)	A (10 cultures, 1 expt.)	0	0. 32	0. 32	0. 32	0. 32
		2	1. 05	1. 75	2. 30	1. 25
		4	2. 75	3. 65	5. 40	4. 75
		6	4. 75	5. 40	5. 70	6. 15
		8	5. 12	6. 15	6. 52	6. 92
	B (30 cultures, 3 expts.)	0	0. 49	0. 49	0. 49	0. 49
		2	2. 03	2. 56	2. 50	2. 61
		4	3. 83	4. 92	5. 41	5. 40
		6	5. 45	5. 26	5. 46	5. 91
		8	6. 10	5. 75	6. 25	6. 95
	C (10 cultures, 1 expt.)	0	0. 92	0. 92	0. 92	0. 92
		2	1. 80	2. 25	2. 50	2. 00
		4	3. 60	4. 00	4. 35	3. 75
		6	5. 75	5. 50	7. 00	6. 00
		8	6. 11	6. 30	7. 52	6. 80
HeLa	A (20 cultures, 2 expts.)	0	0. 42	0. 42	0. 42	0. 42
		2	0. 55	1. 21	1. 60	1. 35
		4	0. 75	1. 75	1. 94	2. 00
		6	1. 12	2. 05	2. 53	3. 20
		8	1. 84	3. 66	3. 41	3. 62

*Initial cell number is an average of all cultures for each condition.
†Each figure represents the results of only 1 experiment.

strain L cells, when the initial cell number averaged 0.45 million per ml. (or per culture), are presented in table 1-A (NCTC clone 929). The figures represent average results for 5 experiments or a total of 50 cultures for each condition.

The beneficial effect of trypsin on strain L cells is marked. In fact, the cells become so numerous that they slough off the glass substratum in great numbers after the 6th or 7th day. For this reason, the experiments were usually discontinued after the 8th day.

The results of the experiment in which the initial cell number averaged 0.65 million per ml. are given in table 1-B (NCTC clone 929). Three experiments were carried out in this range. In general, the results were

similar to those obtained for the experiments in which lower initial cell inoculums were used.

There is a much greater increase in cell numbers produced in strain L cells exposed to concentrations of trypsin, 62.5 mg. percent and below, than in medium containing no trypsin.

NCTC clone 1469 (mouse liver epithelial cells).—Five experiments were carried out with mouse liver cells; these were run in a manner similar to those already reported.

The results obtained when the initial cell count averaged 0.32 million cells per ml. are presented in table 1-A (NCTC clone 1469). The liver cells reproduced rapidly and thus were not carried beyond the 8th day.

Three experiments were conducted with an initial cell number approximating 0.49 million per ml. and 1 experiment was conducted with an initial inoculum of 0.92 million per ml. The results are presented in table 1-A, B, and C (NCTC clone 1469), respectively, and they indicate that very little effect, if any, was exerted on the liver cells, which is contrary to the results obtained with strain L cells.

HeLa strain.—The HeLa cells are epithelial in nature and do not multiply as rapidly as those of strain L, under the conditions in which they were cultivated.

Two experiments were carried out to study the effects of trypsin on these cells. The same techniques and procedures were used as for strain L cells and, just as in the latter, the HeLa cells soon died in concentrations of trypsin, 125 mg. percent or greater. The effects of lower concentrations are recorded in table 1-A (HeLa), which indicate the beneficial effects of trypsin at these concentrations.

Thus, although the effect is not as obvious in HeLa cells as in strain L, there is a definite increase in cell number in the presence of any of the three different concentrations of trypsin. Although the lower concentrations of trypsin result in an increase in cell numbers in strain L and HeLa, there is no indication as to how this is accomplished. Perhaps the effect is on certain constituents of the nutrient medium, or on the cells directly, or on both medium and cells. In an attempt to ascertain the real reason, the following experiments were conducted.

Effect of Trypsin on the Nutrient Medium

The same cell strains—NCTC clone 929, NCTC clone 1469, and HeLa—as well as the same concentrations of trypsin were used as for the previous experiments. Actually, the embryo extract and the horse serum were digested separately at 37° C. for 24 hours preceding the experiment, after which the tryptic activity was "destroyed" by heating to 56° C. for 30 minutes. The same treatment was given to the nutrient medium, used as the control, in which no trypsin was added. The results are compiled in table 2 and are comparable to the results obtained previously for the same cell strains (table 1). Trypsin, in these concentrations, has a definite stimulating effect on the growth of strain L and HeLa cells,

TABLE 2.—Effects of 40:40:20 medium containing horse serum and embryo extract treated separately with different concentrations of trypsin upon cells *in vitro**

Cell type	Experimental group	Age of cultures (*days*)	Concentration of trypsin (*mg. %*)			
			0 (Control)	16	31	62.5
			Total number of cells (*millions per ml.*)			
NCTC clone 929 (Earle's strain L)	A	0	0. 90	0. 95	0. 95	0. 96
		2	0. 96	1. 83	2. 39	1. 90
		4	2. 40	6. 50	7. 00	6. 90
		7	3. 50	6. 80	7. 40	7. 50
	B	0	0. 42	0. 43	0. 44	0. 43
		2	1. 40	2. 90	2. 70	3. 00
		4	2. 50	4. 80	4. 00	5. 00
		7	3. 40	5. 20	6. 00	7. 50
NCTC clone 1469 (mouse liver)	A	0	0. 81	0. 77	0. 85	0. 80
		2	1. 80	1. 20	1. 16	1. 18
		4	2. 50	1. 20	1. 05	0. 95
		7	3. 50	3. 80	0. 95	1. 05
	B	0	0. 58	0. 58	0. 58	0. 58
		2	1. 50	0. 95	1. 20	1. 40
		4	3. 00	0. 85	0. 60	0. 75
		7	5. 50	0. 90	0. 75	0. 80
HeLa	A	0	0. 20	0. 20	0. 20	0. 20
		2	0. 42	0. 35	0. 35	0. 44
		5	0. 63	1. 13	1. 20	1. 20

*Initial cell number is an average of all cultures for each condition. Each initial figure represents an average for 20 cultures (2 expts.).

either when large numbers (0.90 to 0.96 million cells per ml.) are present in the initial inoculums or when smaller numbers (0.42 to 0.46 million cells per ml.) are present.

In both experiments involving mouse liver cells, table 2-A and 2-B (NCTC clone 1469), there is a definite inhibitory effect in 1 of 2 tests with 16 mg. percent trypsin and in all concentrations above 16 mg. percent.

In HeLa cells the effect was similar to that found in strain L, *i.e.*, there was a greater increase in the number of cells, during the period of the experiment, in the cultures containing trypsin than in the controls, although this increase was not as evident as it was in the experiments on strain L (*see* table 2-A, HeLa).

Effect of Trypsin on Horse Serum Added to the Nutrient Medium

The same types of cells were used. In these experiments the horse serum was digested for 24 hours at 37° C., after which tryptic activity was "destroyed" by heating to 56° C. for 30 minutes. The embryo extract was not trypsinized. No trypsin was added to that part of the nutrient medium used as the control, but the control medium was heated to 56° C. for 30 minutes after a 24-hour period at 37° C.

The results are given in table 3 and for the most part coincide with those reported in tables 1 and 2. The difference in final cell numbers for

strain L and HeLa cells was not as great as in the previous tests, but there was a greater increase in cell number in the cultures in medium containing horse serum that had been acted upon by trypsin.

However, in the liver cells there was a definite inhibitory effect as shown in table 2-A and 2-B, NCTC clone 1469. This inhibitory effect was more evident after the 2d day of the experiment.

TABLE 3.—Effects of 40:40:20 medium containing horse serum treated with different concentrations of trypsin upon cells *in vitro*—the embryo extract was not treated with trypsin*

Cell type	Experimental group	Age of cultures (*days*)	Concentration of trypsin used in digesting horse serum (*mg. %*)			
			0 (control)	16	31	62.5
			Total number of cells (*millions per ml.*)			
NCTC clone 929 (Earle's strain L)	A	0	0. 97	0. 95	0. 95	1. 00
		2	1. 00	2. 00	2. 34	2. 00
		4	2. 50	6. 75	5. 75	5. 50
		7	3. 20	7. 20	8. 00	7. 25
	B	0	0. 45	0. 47	0. 45	0. 45
		2	1. 50	2. 80	3. 20	3. 00
		4	2. 50	5. 00	5. 00	6. 00
		7	3. 50	6. 20	6. 50	6. 10
NCTC clone 1469 (mouse liver)	A	0	0. 85	0. 77	0. 82	0. 75
		2	1. 85	1. 05	1. 00	0. 90
		4	2. 30	1. 25	1. 35	1. 30
		7	3. 00	1. 50	2. 00	1. 75
	B	0	0. 56	0. 57	0. 58	0. 56
		2	1. 20	0. 75	0. 80	0. 96
		4	3. 20	0. 65	0. 80	0. 63
		7	5. 25	0. 72	0. 93	0. 70
HeLa	A	0	0. 20	0. 20	0. 20	0. 20
		2	0. 42	0. 30	0. 33	0. 45
		5	0. 63	0. 85	0. 94	0. 63

*Same three types of cells were used as in previous experiments. Initial cell number is an average of all cultures for each condition. Each initial figure represents an average for 20 cultures.

Effect of Trypsin on Chick-Embryo Extract Used in Nutrient Medium

The embryo extract was exposed to trypsin in the same manner as the horse serum in the previous experiment. As usual the nutrient medium used as the control was kept at 37° C. for 24 hours and then heated to 56° C. for 30 minutes.

The results are presented in table 4, and again the effect of trypsinized embryo extract varied, depending upon the type of cell used.

In strain L cells, trypsinized embryo extract resulted in a greater increase in cell number than in cultures not so exposed. Mouse liver cells, however, in trypsinized embryo extract were inhibited in growth similar to that noted in the two previous experiments. Although HeLa cells increased in number, when exposed to trypsinized embryo extract,

TABLE 4.—Effects of 40:40:20 medium containing embryo extract treated with different concentrations of trypsin upon cells *in vitro*—the horse serum portion was not treated with trypsin*

Cell type	Experimental group	Age of cultures (*days*)	Concentration of trypsin used in digesting embryo extract (*mg. %*)			
			0 (Control)	16	31	62.5
			Total number of cells (*millions per ml.*)			
NCTC clone 929 (Earle's strain L)	A	0	0. 97	0. 97	0. 95	0. 90
		2	1. 05	2. 10	2. 20	2. 05
		4	2. 40	4. 65	5. 90	4. 60
		7	3. 50	8. 00	9. 00	6. 25
	B	0	0. 45	0. 42	0. 45	0. 43
		2	1. 40	2. 50	2. 90	2. 90
		4	2. 50	6. 00	5. 00	5. 75
		7	3. 40	6. 50	6. 00	5. 10
NCTC clone 1469 (mouse liver)	A	0	0. 80	0. 80	0. 77	0. 82
		2	1. 75	0. 90	1. 00	1. 10
		4	2. 35	0. 92	1. 00	0. 96
		7	3. 05	0. 85	3. 00	2. 10
	B	0	0. 55	0. 58	0. 55	0. 57
		2	1. 12	0. 72	0. 65	0. 70
		4	3. 00	0. 80	0. 70	0. 85
		7	5. 50	0. 94	0. 83	0. 92
HeLa	A	0	0. 20	0. 20	0. 20	0. 20
		2	0. 42	0. 25	0. 31	0. 38
		5	0. 63	0. 70	0. 78	0. 85

*See footnote, table 3.

to a greater extent than the control cultures, this difference is not significant.

Effect of Trypsin on Cells Cultivated in a Semisynthetic Medium

In this series of experiments only strain L cells were used. These cells were cultivated in a semisynthetic medium consisting of NCTC # 109 [Sanford *et al.* (*22*)] supplemented with 10 percent horse serum. The experimental procedure was similar to that described when cells were cultivated in serum and embryo-extract medium. Under these conditions, concentrations of trypsin 62.5 mg. percent and greater were toxic to the cells; they died within 48 hours. Therefore, the following concentrations of trypsin were used in these experiments: 31, 16, and 10 mg. percent. The initial cell numbers varied from 0.20 to 0.75 million per ml.

Counts were made every 2 days, but only the final 8-day counts (which are considered maximal) are recorded in table 5. Although an exception was found in one of the experiments, cell proliferation was retarded in five other experiments, in media containing 31 mg. percent trypsin. But, very little, if any, effect was found in media containing 15 or 10 mg. per-

cent. This is contrary to the results obtained for cells cultivated in horse serum-extract medium containing various concentrations of trypsin.

These results are verified by photomicrographs that were taken every 2d day of the cells exposed to various trypsin concentrations. Figures 1 through 4 illustrate the condition of the cell populations after 5 days in medium # 109 plus horse serum (fig. 1), and in medium # 109 plus horse serum plus 10 mg. percent trypsin, plus 15 mg. percent trypsin, and plus 31 mg. percent trypsin (figs. 2, 3, and 4, respectively).

Trypsin appeared to be far more toxic to cells in the synthetic medium (NCTC # 109 plus 10% horse serum) than to cells grown in medium containing greater quantities of horse serum. Undoubtedly, this phenomenon is owing to the much lower concentration of antitrypsin that is present in 10 percent as compared to 40 percent serum. Also the results of the study made on the effects of trypsin in low concentration indicated very little, if any, stimulatory effect on cells grown in the semisynthetic medium.

TABLE 5.—Effects of trypsin on strain L cells cultivated in NCTC medium # 109 plus 10 percent *horse serum*. A comparison is made of cell number at the end of 8 days' exposure to trypsin when different initial cell densities are used

	Concentration of trypsin (*mg. %*)			
	0 (control)	10	16	31
Initial cell number	Number of attached cells after 8 days (*millions per ml.*)			
200, 000	3. 06	3. 78	3. 52	1. 11
230, 000	4. 04	4. 97	3. 91	2. 60
250, 000	3. 28	3. 78	3. 81	2. 39
460, 000	3. 80	4. 76	4. 67	4. 32
600, 000	4. 00	4. 68	5. 39	1. 44
750, 000	4. 00	4. 65		3. 18

DISCUSSION

Investigators in the field of tissue culture, or closely related areas, usually depend on trypsin to separate cells and suspend them in the medium for culturing. Trypsin readily digests certain proteins found in intercellular material. There is always the possibility, however, that trypsin may have an effect on the living cell itself, or that once the cell is exposed to trypsin it may no longer respond normally. Fermi (*11*) and others, however, have shown that many living cells can withstand relatively high concentrations of trypsin.

The results of the experiments reported here indicate that the proliferation of cells *in vitro* acted upon by trypsin may be accelerated, inhibited, or unaffected, depending upon the effective concentration of the enzyme in the nutrient medium and/or the type of cell under investigation. By

effective concentration is meant the quantity of trypsin remaining in the medium after inactivation of some portion of the enzyme by factors such as antitrypsin. The effective concentration would also depend upon temperature, *p*H, and available surface area.

Time and temperature are factors which have been controlled in all the experimental conditions used in these investigations. The H-ion concentration is a changeable factor, and though adjusted to *p*H 7.4 in the original medium of each culture, it soon changes toward the acid side at a rate depending mainly on the rate of metabolism of the cells in each culture. The mouse liver cells metabolize comparatively rapidly, thus causing a greater acidity in unit time than either the HeLa or strain L cells. Since the optimum H-ion concentration for tryptic activity is between *p*H 8 to 9, the more rapid increase in acidity in the liver-cell cultures will decrease the tryptic activity. This may be the factor that causes a response in the mouse liver cells quite different from that in strain L or HeLa cells. The latter two strains proliferate to a much greater extent than the liver cells when exposed to 16, 31, or 62.5 mg. percent trypsin. The liver cells show very little, if any, significant effects.

The antitrypsin factor in the serum is found in lower concentration in the synthetic medium, to which only 10 percent horse serum is added, than in the horse serum-embryo extract medium which contains 40 percent horse serum. This is apparently the reason why cells cultivated in the latter medium are able to withstand higher concentrations of trypsin than cells cultivated in the synthetic medium with a lower concentration of horse serum.

The beneficial effects of trypsin, insofar as maximum cell numbers (strain L and HeLa) are concerned, are evidently due to the partial or complete digestion of the protein of horse serum and to the embryo extract in the 40:40:20 medium. This also applies to the effects of the medium when either horse serum or embryo extract is predigested by trypsin. The results obtained when trypsin was added to the medium containing only 10 percent horse serum verify this. Evidently the products produced by the digestion of this lesser quantity of protein are insufficient to produce more than a mere suggestion of an increase in proliferation in strain L cells in 10 or 16 mg. percent trypsin.

The mouse liver cells were affected very little, if at all, when trypsin (62.5 mg. % and below) was added to the 40:40:20 medium, and proliferation was actually inhibited when they were exposed to a medium containing "predigested" protein of horse serum or embryo extract. It will be necessary to carry these investigations further so that a more complete understanding and interpretation of the results will be possible.

The higher toxicity of trypsin observed in the semisynthetic medium plus 10 percent horse serum is, undoubtedly, indirectly due to the lower antitrypsin content in this medium, as compared to that in the 40:40:20 medium. Thus, there is a higher effective concentration of trypsin in the semisynthetic medium. This indicates that the toxic effect of trypsin is evidently due to its direct action on the cells.

REFERENCES

(1) ROUS, P., and JONES, F. S.: A method for obtaining suspensions of living cells from the fixed tissues and for the plating out of individual cells. J. Exper. Med. 23: 549–555, 1916.

(2) YOUNGNER, J. S.: Monolayer tissue culture. I. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. Proc. Soc. Exper. Biol. & Med. 85: 202–205, 1954.

(3) RINALDINI, L. M. J.: The isolation of living cells from animal tissues. Internat. Rev. Cytol. 7: 587–647, 1958.

(4) MOSCONA, A.: Cell suspensions from organ rudiments of chick embryos. Exper. Cell Res. 3: 535–539, 1952.

(5) ———: Development of heterotypic combinations of dissociated embryonic chick cells. Proc. Soc. Exper. Biol. & Med. 92: 410–416, 1956.

(6) MOSCONA, A., and MOSCONA, H.: The dissociation and aggregation of cells from organ rudiments of the early chick embryo. J. Anat. 86: 287–301, 1952.

(7) FRISCH, A. W., and JENTOFT, V.: Use of trypsin in preparing subcultures of monkey testicular tissue. Proc. Soc. Exper. Biol. & Med. 82: 322–323, 1953.

(8) CHANG, R. S. M.: Continuous subcultivation of epithelial-like cells from human normal tissues. Proc. Soc. Exper. Biol. & Med. 87: 440–443, 1954.

(9) SIMMS, H. S., and STILLMAN, N. P.: Substances affecting adult tissue in vitro. I. The stimulating action of trypsin on fresh adult tissue. J. Gen. Physiol. 20: 603–619, 1936.

(10) ———: Substances affecting adult tissue in vitro. II. A growth inhibitor in adult tissue. J. Gen. Physiol. 20: 621–629, 1936.

(11) FERMI, C.: Sur les moyens de defense de l'estomac, de l'intestin, du pancréas et en géneral de la cellule et de l'albumine vivante vers les enzymes protéolytiques. Centr. f. Bacteriol. u. Parisiten Abt. I. Orig. 56: 55–85, 1910.

(12) MEDAWAR, P. B.: Sheets of pure epidermal epithelium from human skin. Nature, London 148: 783, 1941.

(13) RUNNSTROM, J., MONNE, L., and BROMAN, L.: On some properties of the surface layer of immature and mature sea urchin eggs and their changes upon activation. Ark. Zool. 35A, 3: 1, 1944.

(14) NORTHROP, J. H.: Action of papain and ficin on tadpoles and arbacia eggs. J. Gen. Physiol. 30: 375–376, 1947.

(15) MEYER, K., and RAPPORT, M. M.: The mucopolysaccharides of the ground substance of connective tissue. Science 113: 596–599, 1951.

(16) LEVAN, A., and BIESELE, J. J.: Role of chromosomes in cancerogenesis as studied in serial tissue culture of mammalian cells. Ann. New York Acad. Sc. 71: 1022–1053, 1958.

(17) MAGEE, W. E., SHEEK, M. R., and SAGIK, B. P.: Methods of harvesting mammalian cells grown in tissue culture. Proc. Soc. Exper. Biol. & Med. 99: 390–392, 1958.

(18) NORTHROP, J. H.: The resistance of living organisms to digestion by pepsin or trypsin. J. Gen. Physiol. 9: 497–502, 1926.

(19) PACE, D. M., and AFTONOMOS, L.: Effects of cell density on cell growth in a clone of mouse liver cells. J. Nat. Cancer Inst. 19: 1065–1075, 1957.

(20) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E.: The measurement of proliferation in tissue culture by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(21) EARLE, W. R., SANFORD, K. K., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The influence of inoculum size on proliferation in tissue cultures. J. Nat. Cancer Inst. 12: 133–153, 1951.

(22) SANFORD, K. K., MCQUILKIN, W. T., FIORAMONTI, M. C., EVANS, V. J., and EARLE, W. R.: Study of amino acid requirements for increase in cell population of NCTC clone 929 (strain L). J. Nat. Cancer Inst. 20: 775–786, 1958.

PLATE 43

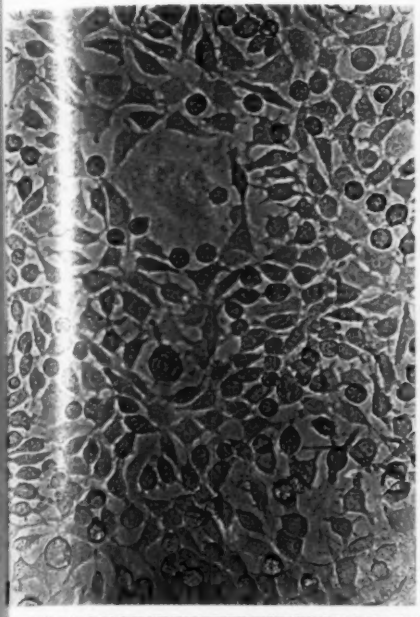
Photomicrographs of strain L cells after 5 days' exposure to different concentrations of trypsin. Initial cell number, 230,000 cells per ml.

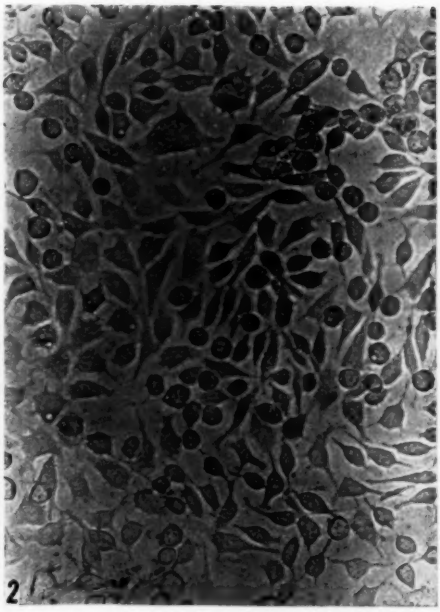
FIGURE 1.—Cells in NCTC #109 plus 10 percent horse serum.

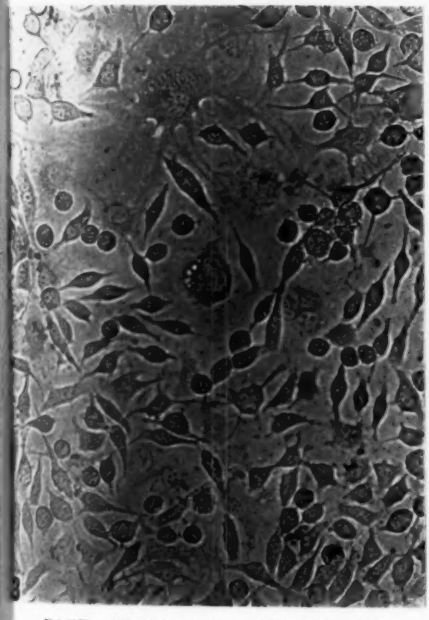
FIGURE 2.—Cells in #109 plus 10 percent horse serum plus 10 mg. percent trypsin.

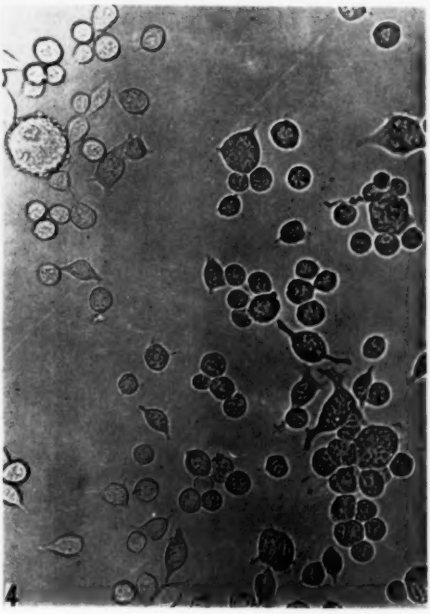
FIGURE 3.—Cells in #109 plus 10 percent horse serum plus 15 mg. percent trypsin.

FIGURE 4.—Cells in #109 plus 10 percent horse serum plus 31 mg. percent trypsin.









511629—59——5

Attempts to Reproduce Cirrhosis of the Liver in Rats After Total-Body X Irradiation [1, 2]

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SUMMARY

Rats fed a low-protein (6% casein) diet in these experiments had fatty infiltration and varying degrees of fibrous proliferation of the liver regardless of whole-body X irradiation. Severity of the fibrotic lesions was reduced by choline supplementation. The degree of severity of fibrous proliferation also seemed to be dependent on the age of the rats when placed on the low-protein diet. Fibrosis was seen generally in the animals with the most severe degree of fat infiltration.—J. Nat. Cancer Inst. 23: 669–673, 1959.

IT HAS been reported (*1*) that rats maintained on a 6-percent protein (casein) diet develop gross and microscopic cirrhotic lesions of the liver after exposure to 450–500 r of X irradiation. The results suggest that superposition of suitable stresses upon test animals brings about the unmasking of injury in an organ that has been reported, by others (*2, 3*), to be quite resistant to radiation damage. The experiments reported (*1*) are of importance not only in describing lesions in a specific organ in post-irradiation rats, on prolonged feeding of a low-protein diet, but also in providing a basis for experiments designed to assess injury of an organ after an extended period during which the test animals have apparently recovered from the radiation episode.

The present communication deals with attempts to reproduce the important experiment previously reported (*1*) and to amplify it so as to assess the latent injury after apparent radiation recovery.

EXPERIMENTAL PROCEDURE

Weanling male and female rats (169 in all) of the Sprague-Dawley [4] strain born and reared in the laboratory colony were used in this investiga-

[1] Received for publication March 20, 1959.

[2] The opinions and assertions contained in this report are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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[4] Personal communication from Dr. J. White states that the Sprague-Dawley rats he used weighed 110 to 120 gm. at 7 to 8 weeks of age. Those used here averaged 210 gm. for males and 165 for females at that age.

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tion. The rats were all placed on the experimental diet shown in table 1, and grouped according to age and radiation-exposure patterns described in table 2. Radiation exposures were made with a General Electric Maxitron Unit operating at 250 kvp, 25 ma., HVL 2.7 mm. Cu, and target skin distance (TSD) of 115 cm. The rats were placed in the beam in

TABLE 1.—Composition of experimental diet*

	Grams
Casein (crude)	60
Sucrose	150
Inorganic salts (USP XIV)	40
Cornstarch	475
Crisco	240
Yeast (Brewer's)	20
Cod-liver oil	10
Cystine	5
Total	1, 000

*All the rats were on a Purina chow diet until placed on this experimental diet.

TABLE 2.—Grouping of rats in experimental design according to age when placed on experimental diet, and radiation-exposure pattern

Group No.*		Number of rats	Sex	Age in weeks when placed on experimental diet	Age in weeks when X ray began	Exposure pattern	Total X-ray dose
I	(X)	10	M	3	7	Single exposure	475 r
		10	F				
	(C)	10	M				
		10	F				
II	(X)	10	M	3	7	200 r/week†	1000 r
		10	F				
	(C)	10	M				
		10	F				
III‡	(X)	8	M	3	7	200 r/week†	1000 r
		7	F				
	(C)	8	M				
		7	F				
IV	(X)	12	M	7	7	200 r/week†	1000 r
	(C)	7					
V	(X)	12	M	12	7	200 r/week†	1000 r
	(C)	8					
VI	(X)	12	M	19	7	200 r/week†	1000 r
	(C)	8					

*X = irradiated; C = control.

† The 200 r per week was administered for 5 consecutive weeks.

‡ This group received an additional vitamin supplement in mg. which provided per rat per day: thiamine hydrochloride, riboflavine, folic acid, and pyridoxine hydrochloride, each 0.02; calcium pantothenate and *p*-amino benzoic acid, each 0.1; nicotinamide, 0.066; inositol, 2.5; biotin, 0.002; and choline, 10.0.

3-tiered individual plastic boxes in which the dose rate in air, monitored with a Victoreen ionization chamber before and after each run, was 13 r per minute.

All the irradiated rats were allowed food and water *ad libitum*. Pair feeding was done on control, unirradiated litter mates until it was observed that food intake of the irradiated rats had returned to pre-exposure levels, *i.e.*, in a period of 2 to 3 days. All the animals were continued on the experimental regimen until death or until killed at 283 days after the last radiation exposure. At the time the rats died or were killed the livers were examined grossly and small pieces, always from the left lateral lobe, were fixed in 15 percent formalin, sectioned, and then stained with hematoxylin and eosin. Sections from each piece of tissue were also stained with phosphotungstic acid and iron hematoxylin, which is specific for fibrous tissue.

Criteria for Morphologic Classification on the Basis of Microscopic Examination

Fatty infiltration.—The grading was done on the basis of subjective impression of the amount of vacuolization present in the liver. Verbal ratings assigned were none to slight, moderate, and severe.

Fibrous proliferation.—Ratings were designated here, as previously, none to slight—no vascular changes, very slight bands of connective tissue, and no, or minimal, parenchymal involvement. Moderate—thickened bands of connective tissue, some vascular damage, and up to 50 percent of parenchymal involvement. Severe—extensive vascular damage with 50 to 100 percent of the parenchymal tissue isolated into nodules.

RESULTS

During the first 70 days after radiation exposure, 8 rats died. Five of the 8 were irradiated and 3 were controls. The 3 controls had gross nodular cirrhosis. The 5 irradiated rats showed no gross lesions but did fall in the none-to-slight grouping when examined microscopically. All 8 of these animals showed severe fatty infiltration.

In the intervening 210 days before all the animals were killed, 15 more rats from all groups died. Autolysis of some of the livers precluded the taking of specimens, but among those examined, the irradiation appeared to have no influence on the onset of cirrhosis.

Histologic results are tabulated in table 3 according to the criteria outlined. As no differences were noted between the sexes, the results have all been combined in this table.

Examination of the results in table 3 showed that (1) irradiation, regardless of total dose or pattern of exposure, caused no increase of fibrous proliferation over that seen in the controls; (2) vitamin supplementation of the diet in group III reduced the fatty infiltration to some

TABLE 3.—Fibrous proliferation and fatty infiltration in the liver after various radiation-exposure patterns and dietary regimens

Group		Number of rats	Fibrous proliferation: Degree of lesion			Fatty infiltration: Degree of lesion		
			Severe	Moderate	Slight or none	Severe	Moderate	Slight or none
I	(X)	18	3	3	12	17	1	0
	(C)	17	6	4	7	17	0	0
II	(X)	19	0	5	14	12	7	0
	(C)	18	4	2	12	15	3	0
III	(X)	13	0	0	13	8	5	0
	(C)	14	0	0	14	4	5	5
IV	(X)	11	0	0	11	9	2	0
	(C)	7	0	0	7	7	0	0
V	(X)	9	0	0	9	5	3	1
	(C)	6	0	0	6	3	3	0
VI	(X)	8	0	1	7	7	0	1
	(C)	5	0	0	5	3	0	2

degree and all but eliminated fibrous proliferation; (3) the minimal cirrhosis observed in groups IV, V, and VI indicates the possibility of an age dependence in the production of these lesions; (4) the fibrosis was seen in the animals with the most severe degree of fatty infiltration.

DISCUSSION

Evidence is accumulating that animals that have sustained a radiation exposure and survived appear to recover to a degree which makes them superficially indistinguishable from controls. Such animals, however, have a reduced life expectancy (*4*) and perhaps other physiologic and biochemical incompetency. The results reported previously (*1*) suggested to the present authors that superposition of a low-protein diet on "apparently recovered" animals might prove useful in unmasking injury which otherwise might not appear.

The experiments reported here were an attempt to reproduce those of White (*1*) and were extended in an effort to show that injury sustained by an organ could be unmasked after recovery from acute radiation illness. The radiation-exposure patterns in these experiments were designed to repeat those reported before (*1*) and to subject the animals to a maximal total dose with minimal deaths. The rats were placed on the experimental diet in such a way as to begin superposing the stress of the low-protein diet on animals before exposure, during exposure, during

recovery, and after apparent recovery was complete. Furthermore, the additional vitamin supplementation of one group was intended to preclude the possibility of deficiencies of lipotropic factors.

Attempts to repeat the results, previously presented (*1*), in the experiments reported here were obviously unsuccessful. The results reported earlier (*1*) showed cirrhosis in 53 percent of irradiated survivors with no cirrhosis in controls. Utilizing a 6-percent casein diet with the same or greater total exposure, the rats in the experiments reported here developed the lesions regardless of radiation exposure, and this suggests that the lesions are of dietary origin. The latter conclusion seems to be supported by experiments reported by others (*5*, *6*) who have shown that a diet similar to the one used by White (*1*), and in the present report, but containing 8 percent instead of 6 percent casein, caused cirrhosis of the liver in rats in 150 days. Cystine in 5-mg. quantities per day aggravated the condition. In the diet used here and previously (*1*), the level of cystine was such that 10 gm. of food per day provided 50 mg. of cystine.

Although the diet composition used here was the same as that reported by White (*1*), it is very doubtful that the dietary ingredients were all procured from the same source. Crude casein is the one ingredient in the diet that might tend to vary a great deal, from several sources, due to its variability in quantity of total nitrogen, from one lot to another. This variability could cause discrepancies among reports from different laboratories and would seem especially possible when one is working with diets containing no more than 6 percent casein. Although the strain of rats used here and previously (*1*) were nominally the same, isolation of substrains from the parental strain, for some time, could lead to differences in cirrhotogenic tendencies due possibly to unconscious selective breeding.

REFERENCES

(*1*) White, J., Congdon, C. C., David, P. W., and Ally, M. S.: Cirrhosis of the liver in rats following total-body X irradiation. J. Nat. Cancer Inst. 15: 1155–1163, 1955.

(*2*) Furth, J., and Upton, A. C.: Vertebrate radiobiology: histopathology and carcinogenesis. Ann. Rev. Nuclear Sc. 3: 303–338, 1953.

(*3*) Patt, H. M., and Brues, A. M.: The pathological physiology of radiation injury in the mammal. II. Specific aspects of the physiology of radiation injury. *In* Radiation Biology, New York, McGraw-Hill Book Co., Inc., 1954, vol. 1, Part 2, Chapt. 15, pp. 986–988.

(*4*) Furth, J., Upton, A. C., Christenberry, K. W., Benedict, H., and Moshman, J.: Some late effects in mice of ionizing radiation from an experimental nuclear detonation. Radiology 63: 562–570, 1954.

(*5*) Webster, G.: Dietary liver disease in rats. (Abstract.) J. Clin. Invest. 20: 440, 1941.

(*6*) Earle, D. P., jr., and Victor, J.: Cirrhosis of the liver caused by excess dietary cystine. J. Exper. Med. 73: 161–172, 1941.

Effect of Iodine on Thyroid Tumors Induced in the Rat by a Low-Iodine Diet [1, 2]

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SUMMARY

Neoplastic thyroid nodules were induced in rats by feeding them a low-iodine diet for 1 year. Then the rats were supplemented with iodine for 2, 4, 8, 16, 32, and 64 days, respectively, and killed. Controls not receiving iodine were killed at the same intervals. The number and the volume of the thyroid nodules were estimated quantitatively. Iodine treatment produced a reappearance of thyroid hormone in the blood and brought the level of thyrotrophic hormone back to a normal range. It caused a regression in the size of the thyroid gland and a considerable increase in the number of "light cells." Two main types of nodules were observed; namely, type-β, formed of polarized cells having some resemblance to the cells lining the lumen of the thyroid follicles and probably originating from these cells, and type-γ, a solid mass of unpolarized cells probably originating from the "light cells" of the thyroid. Except, perhaps, for the very small ones, most type-β nodules persisted throughout the 2-month iodine treatment period, but they decreased in size. Iodine strongly stimulated the formation of type-γ nodules; after 2 months there were twice as many nodules in the iodine-treated group as in the controls. The formation of the new nodules probably resulted from a progressive growth of the light cells accompanied by a progressive disappearance of follicular cells.—J. Nat. Cancer Inst. 23: 675–693, 1959.

IT HAS been shown that prolonged feeding of a low-iodine diet to rats results in goitrous thyroid glands which contain neoplasms (*1–3*). What is the fate of these neoplasms when the iodine deficiency condition is discontinued? This problem was investigated by examining the thyroid glands of rats kept iodine deficient for 1 year and then given iodine for various lengths of time.

MATERIALS AND METHODS

Four hundred and eighteen female, 3-month-old Holtzman albino rats (descended from a Sprague-Dawley strain) weighing 250 gm., on the

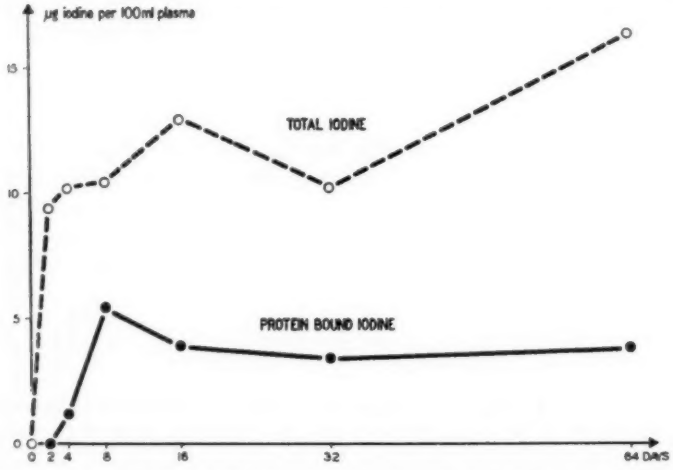
[1] Received for publication March 20, 1959.

[2] This work was supported by a grant from the National Cancer Institute of Canada.

[3] Research Associate of the National Cancer Institute of Canada. The author is indebted to Dr. C. P. Leblond for his advice and criticism, and to Dr. M. Stux for the TSH measurements.

average, were fed a diet consisting of 70 percent corn meal, 16 percent wheat gluten, 10 percent yeast, 2 percent extracted liver residue, 1 percent calcium carbonate, and 1 percent sodium chloride. The iodine content of this diet, estimated according to a modification of the Chaney method (*4*), ranged from 1.7 to 4.9 and averaged 2.9 μg. of iodine per 100 gm. of diet, which is about one seventh of the critical level maintaining morphologically normal thyroid glands (*5*). The animals, which were kept in metal cages provided with wire-mesh floors (2 to 3 rats per cage), received the diet and distilled water for 364 days. At the end of this period, there were 234 rats weighing 364 gm., on the average. They were divided into two equal groups; both groups were kept on the same diet, but one of them was given a sodium iodide solution containing 2 μg. iodine per ml., as drinking water, while the control group was kept on distilled water. In each group, 10 to 12 rats were killed after 2, 4, 8, 16, and 32 days, and 40 to 46 rats after 64 days of iodine treatment. When they were killed, blood was taken from the abdominal aorta and pooled for iodine (total and protein-bound) as well as thyrotrophic hormone (TSH) estimation. Protein-bound and total iodine were measured in the plasma (citrated) according to a modification of the Barker dry-ashing method (*6*). TSH was measured by the method of Del Conte (*7*) which involves the isolation of the TSH-containing fraction of blood by precipitation with acetone, injection of this fraction into guinea pigs (5, per extract), and counting the number of intracytoplasmic colloid droplets in the thyroid gland.

To determine the number and size of the thyroid neoplasms (hereafter referred to as nodules),the thyroid glands of all rats were fixed in Bouin's fixative and sectioned serially (6 μ). Every 50th section was stained with Masson's light-green trichrome and examined under the microscope;



TEXT-FIGURE 1.—Effect of iodine treatment on protein-bound and total iodine levels in rats previously fed a low-iodine diet for 1 year.

thus the distance between two sections examined was 300 μ. The approximate volume of each nodule (assumed to be an ellipsoid) was calculated as previously (*3*) from the diameters in 3 dimensions. Two of the diameters were obtained from measurements made in each section and the third from the number of sections containing the nodule.

The volume of the thyroid gland was determined by estimating the area occupied by thyroid tissue in the series of sections and multiplying this figure by 0.3 mm., that is, the distance between two sections. The area itself was estimated by cutting out the thyroid tissue areas from photographic prints of the slides, weighing them, and comparing them to the weight of a standard area of the same photographic paper.

Statistical evaluation of percentage data was done according to the X^2 test (*8*). Best fit lines were calculated according to the least square criterion (*9*) and significance of their slope was tested by the *t* test (*9*).

RESULTS

The animals that had been on the low-iodine diet for 1 year or more had no, or barely measurable, organic and inorganic iodine in their blood (table 1). After 2 days of iodine treatment, the iodine level was high (9.4 μg./100 ml. plasma), yet no protein-bound iodine was detectable. A small amount of protein-bound iodine (1.2 μg./100 ml.) was found after 4 days. This amount was increased to 5.5 μg. per 100 ml. at 8 days and remained at about 4 μg. per 100 ml. from 16 days on (table 1 and text-fig. 1).

TABLE 1.—Effect of iodine on blood iodine and thyrotrophic hormone in rats previously fed a low-iodine diet for 1 year

Iodine treatment (*days*)	Treatment	Number of rats	Final body weight (*gm.*) Mean ± S.E.	Blood iodine		Blood TSH cytological coefficient* Mean ± S.E.†	Thyroid volume (*mm.*^{3}) Mean ± S.E.
				Total (*μg./100 ml.*)	PBI (*μg./100 ml.*)		
	Controls	11	374 ± 30	0	0	20.6 ± 2.2	151 ± 16
2	Iodine	10	370 ± 31	9.4	0	15.2 ± 4.1	*113* ± *8*
	Controls	10	344 ± 29	0.9	0	24.5 ± 1.5	159 ± 20
4	Iodine	11	357 ± 28	10.2	1.2	15.8 ± 4.6	117 ± 12
	Controls	10	373 ± 30	0	0.8	31.3 ± 4.5	132 ± 14
8	Iodine	10	363 ± 25	10.4	5.5	*14.6* ± *3.6*	102 ± 15
	Controls	12	370 ± 29	0	0.6	24.9 ± 5.8	175 ± 18
16	Iodine	11	352 ± 29	12.9	3.9	*4.9* ± *1.5*	*51* ± *5*
	Controls	10	366 ± 32	0.2	0.5	20.6 ± 3.7	153 ± 20
32	Iodine	11	361 ± 31	10.2	3.4	*5.9* ± *0.8*	*34* ± *7*
	Controls	40	360 ± 11	0	0	—	191 ± 14
64	Iodine	46	350 ± 8	16.3	3.8	—	*39* ± *3*

*Number of colloid droplets per unit follicle diameter.

†Obtained in 4 to 5 guinea pigs. The italicized values (blood TSH and thyroid volume) are significantly different from the respective controls.

The TSH level was high in the iodine-deficient animals (cytological coefficient, 20 to 31). In the iodine-treated rats this level decreased and showed the first significant drop after 8 days ($0.01 < P < 0.02$), a more drastic decrease during the 8 following days, and at the 16th day the cytological coefficient was only about 5 (table 1). This is close to the value of 3.9 obtained in uninjected guinea pigs, which corresponds to TSH level 0. Thus, iodine treatment produced a reappearance of thyroid hormone and a drastic diminution of TSH in the blood.

Iodine treatment did not significantly change the body weight (table 1) and mortality (11.4% in control and 4.8% in iodine-treated rats), but reduced the size of the thyroid gland from about 150 $mm.^3$ to about 35 $mm.^3$ A slight but significant reduction was noted after 2 days, but the diminution occurred mostly during the 2d week of iodine treatment (table 1).

Histological examination of the thyroid glands revealed the presence of numerous nodules. As in previous work (*1–3*), most of these nodules could be classified in two main categories, referred to as type-β and type-γ nodules, respectively.

Effect of Iodine on the Diffuse Goitrous Tissue

The thyroid glands of the rats kept iodine-deficient throughout the experiment were similar to those described earlier (*1–3*). Histologically, they had the characteristic appearance of simple goiter; that is, hyperemia, and almost no colloid and hypertrophic cells, referred to as type-α cells (fig. 2).

After the rats had been given iodine for 2 days, the amount of colloid in the lumen was slightly increased and stained more intensely than in untreated controls. Continuation of the iodine treatment resulted in a further increase in the amount of colloid and in a diminution of cell size. After 2 months, the round-shaped colloid masses were lined with flat cells containing a densely stained nucleus. The follicles were small (fig. 3) and most of them were filled with colloid, which stained green with Masson's light-green trichrome method. Connective tissue was abundant. The "light cells" were numerous in most of the rats. It is recalled that in the normal thyroid gland of old rats, two types of epithelial cells are observed (*1*): (*a*) The simple epithelium-cell type, which is in contact with the colloid, may contain colloid droplets and displays the terminal bars at the line of junction of two neighboring cells, with the terminal web at their surface directed toward the lumen. (*b*) The so-called "light cells" (fig. 1), which are seen usually in small groups and which lie between the first cell type and the basement membrane; these cells are larger, with a larger nucleus and a lightly stained cytoplasm and do not contain colloid droplets or terminal bars. They are probably epithelial in nature (*10*) but their function is not known. Under normal conditions these cells are relatively few (fig. 1). After the period of iodine deficiency followed by iodine treatment, the light cells were much more numerous (fig. 4). This increase in light cells was apparent 8 days after iodine

supplement was started and became more evident at later intervals. In some areas in the gland they occupied as much space as or more than the simple epithelial-cell type. The latter often showed pyknotic nuclei packed against each other and not well oriented, and very little cytoplasm.

Effect of Iodine on Type-β Nodules

Effect on morphology.—The type-β nodules in the thyroid gland of the iodine-deficient rats were the same as described previously (*1–3*). They consisted usually of a simple epithelium in contact with a lumen and a basement membrane. The cells were narrow and the nuclei closely packed side by side. The amount of cytoplasm was less than in type-α cells, but in some of the nodules the cells were fairly high (fig. 5), and in others they were very low (fig. 6).

In the course of iodine treatment, the volume of type-β cells decreased in the same way as that of type-α cells, so that after 1 to 2 months, only a little cytoplasm was present in all type-β nodules (fig. 7).

Effect on incidence and number.—The number of rats with type-β nodules was not changed significantly by iodine treatment (table 2). In both

TABLE 2.—Effect of iodine on type-β nodules

Iodine treatment (*days*)	Treatment	Number of rats	Number of type-β nodules per rat Mean ± S.E.	Rats with type-β nodules	
				Number	Percent
	Controls	11	2.45 ± 0.73	9	82
2	Iodine	10	1.50 ± 0.54	6	60
	Controls	10	1.60 ± 0.40	2	20
4	Iodine	11	1.36 ± 0.45	6	55
	Controls	10	1.80 ± 0.42	8	80
8	Iodine	10	1.50 ± 0.31	8	80
	Controls	12	1.17 ± 0.24	10	83
16	Iodine	11	1.55 ± 0.31	9	82
	Controls	10	2.00 ± 0.65	7	70
32	Iodine	10	1.40 ± 0.37	8	80
	Controls	40	4.60 ± 0.53	37	93
64	Iodine	46	1.89 ± 0.24*	38	83

*Significantly different from control.

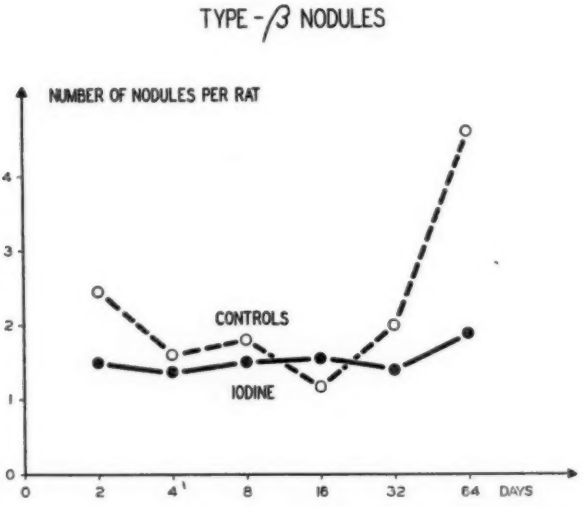
control and iodine-treated groups, the regression lines (% rats with nodules *vs.* log time) were almost identical and indicated an increase from about 57 percent at 2 days to 87 percent at 64 days. This increase with time was significant (regression coefficient: $0.02 < P < 0.05$) in the iodine-treated group; in controls, only the increase in incidence observed during the last month was significant.

The average number of type-β nodules was about the same in iodine-treated as in control rats, during the first month—perhaps slightly lower in the iodine-supplemented group. In the rats not supplemented with iodine, the nodules sharply increased in number during the last

month, while in the iodine-treated group they did not. Thus, iodine prevented this increase (text-fig. 2).

Effect on size.—Since the nodules ranged from 0.001 to 100 mm.^{3}, the average volume would not have been a reliable assessment of their size. Thus type-β nodules were divided into two-size classes, namely, those larger and those smaller than 0.1 mm.^{3} For each class, the number of nodules was plotted *versus* the logarithm of time and the best fit line was calculated (text-fig. 3).

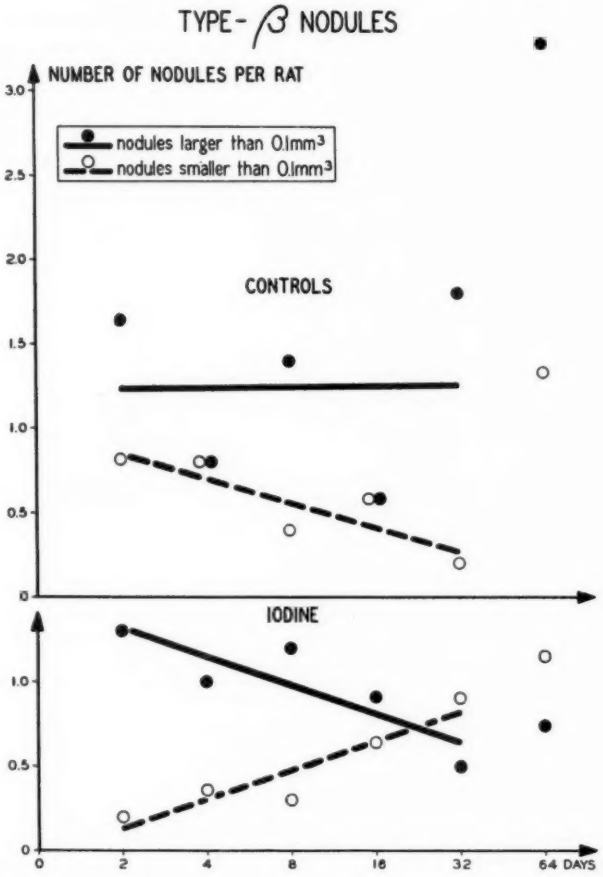
After 2 days of treatment with iodine, the number of nodules smaller than 0.1 mm.^{3} was only one fourth that in controls ($0.05 < P < 0.1$), while the number of nodules larger than this size was not changed (text-fig. 3). When iodine treatment was prolonged, more small nodules and



TEXT-FIGURE 2.—Effect of iodine on the number of type-β thyroid nodules. Iodine treatment was started at time 0 and lasted 2 to 64 days. Iodine prevented the increase in nodules which occurred in controls between 32 and 64 days.

fewer large ones were present. Since the total number of nodules remained the same (text-fig. 2), this indicates that the large nodules decreased in size. Thus, iodine treatment produced a progressive decrease in size of the large nodules and possibly the disappearance of small type-β nodules during the first few days.

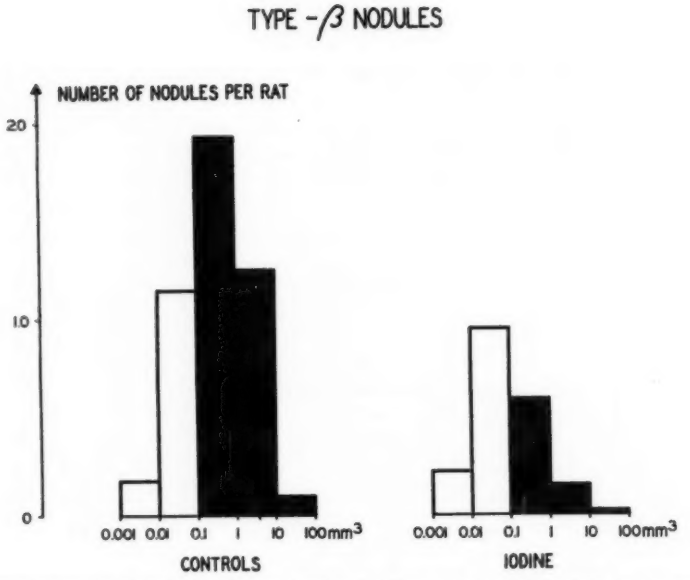
At 64 days, the rats were more numerous and the number of nodules was large enough to allow their distribution into 5-size classes. This more detailed size distribution showed that, after 2 months, the large nodules were much fewer in the iodine-supplemented rats, while the small nodules were present in about the same number as in controls (text-fig. 4). This observation is consistent with the conclusion that iodine produced a regression in the size of type-β nodules.



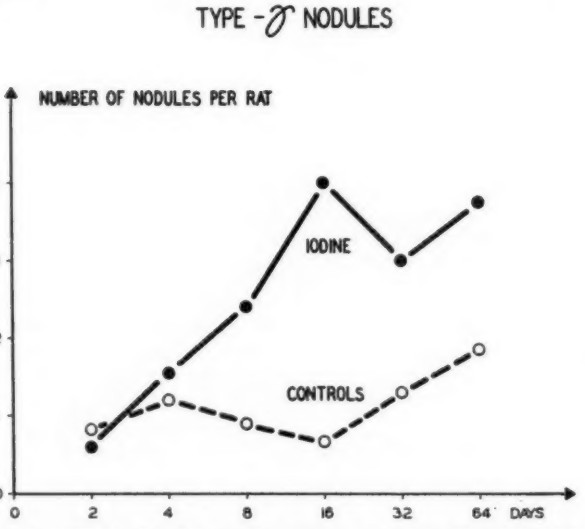
TEXT-FIGURE 3.—Effect of iodine on the size of type-β thyroid nodules. Iodine treatment was started at time 0 and lasted 2 to 64 days. The nodules are divided into two-size classes, namely, those smaller and those larger than 0.1 mm.^{3} At 2 days the small nodules were much fewer in iodine-treated rats than in controls. Between 2 and 64 days, iodine produced an increase in small nodules and a decrease in large nodules. The straight lines are the best fit lines according to the least-square criterion. They were computed from the 2- to 32-day figures only, since later the trend changes in controls (text-fig. 2). P for regression coefficients: controls, large nodules >0.9; controls, small nodules 0.001 to 0.01; iodine, large nodules 0.05 to 0.1; iodine, small nodules 0.02 to 0.05.

Effect of Iodine on Type-γ Nodules

Effect on morphology.—The type-γ nodules in iodine-deficient animals consisted of solid masses of unpolarized cells containing occasional isolated follicles (fig. 8) as described previously (*1–3*). In iodine-treated rats, type-γ nodules showed no apparent cytological differences, but



TEXT-FIGURE 4.—Effect of iodine on the size distribution of type-β nodules in the animals killed after 64 days of iodine treatment. The height of a block indicates the number, per rat, of nodules falling into a given size range, which is indicated in $mm.^3$ on the abscissa. While the number of small nodules (white blocks) was about the same in both groups, the large nodules (black blocks) were much fewer in the iodine-treated rats.



TEXT-FIGURE 5.—Effect of iodine on the number of type-γ thyroid nodules. Iodine treatment was started at time 0 and lasted 2 to 64 days. Iodine produced a considerable increase in the number of nodules.

TABLE 3.—Effect of iodine on type-γ nodules

Iodine treatment (*days*)	Treatment	Number of rats	Number of type-γ nodules per rat Mean ± S.E.	Rats with type-γ nodules	
				Number	Percent
	Controls	11	0. 82 ± 0. 32	5	45
2	Iodine	10	0. 60 ± 0. 22	5	50
	Controls	10	1. 20 ± 0. 36	6	60
4	Iodine	11	1. 55 ± 0. 34	9	82
	Controls	10	0. 90 ± 0. 35	5	50
8	Iodine	10	2. 40 ± 0. 56*	10	100
	Controls	12	0. 67 ± 0. 22	6	50
16	Iodine	11	4. 00 ± 0. 69*	10	91
	Controls	10	1. 30 ± 0. 47	6	60
32	Iodine	10	3. 00 ± 0. 82	7	70
	Controls	40	1. 85 ± 0. 24	30	75
64	Iodine	46	3. 57 ± 0. 34*	46	100

*Significantly different from control.

isolated follicles or follicle remnants were more frequent and more numerous (fig. 9).

Effect on incidence and number.—The proportion of rats with type-γ nodules increased with time in iodine-treated rats and in controls, although somewhat faster in the former than in the latter. Thus, at 16 and 64 days, the proportion of animals with nodules was significantly larger in the iodine-treated group than in controls (table 3).

The average number of type-γ nodules per rat increased considerably with time in iodine-treated rats, while in controls it rose only slightly and at 64 days there were twice as many nodules in the iodine-treated group as in controls (text-fig. 5) ($P<0.001$).

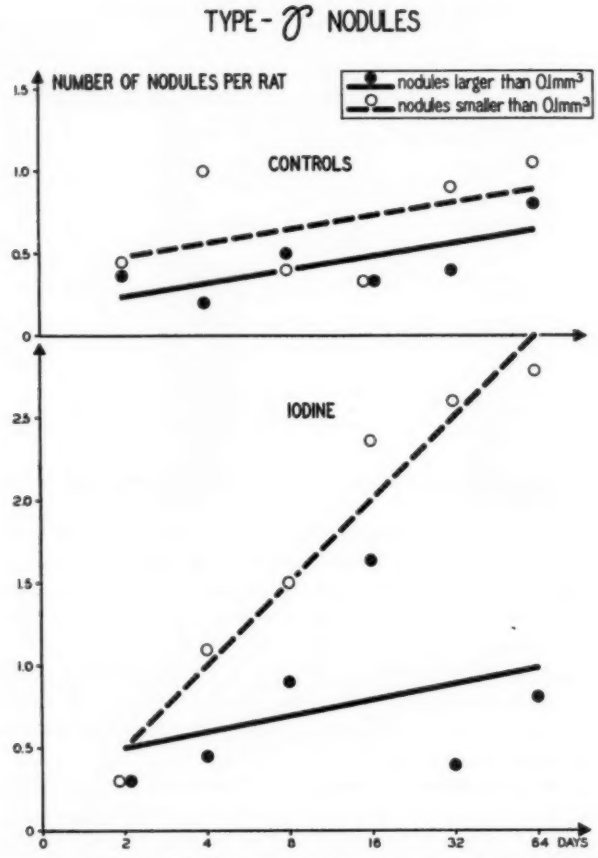
Thus, iodine treatment increased the incidence and the number of type-γ nodules.

Effect on size.—When type-γ nodules were divided into 2-size classes, namely, those smaller and those larger than 0.1 mm.^{3}, it became apparent that iodine treatment had stimulated the production of the small nodules only, while the rate of increase in the large ones was the same as in the controls (text-fig. 6). After 2 months there were about 3 times as many small nodules in the iodine-treated group as in the controls. The same observation was made when the nodules of the animals killed at 64 days were distributed into 5-size classes (text-fig. 7).

Thus, iodine stimulated the production of type-γ nodules, and these newly formed nodules were small.

DISCUSSION

Type-β nodules.—There is strong evidence that the production of type-β thyroid nodules by the low-iodine diet is due to prolonged thyrotrophic stimulation: (1) The rats on the low-iodine diet had a high level of cir-



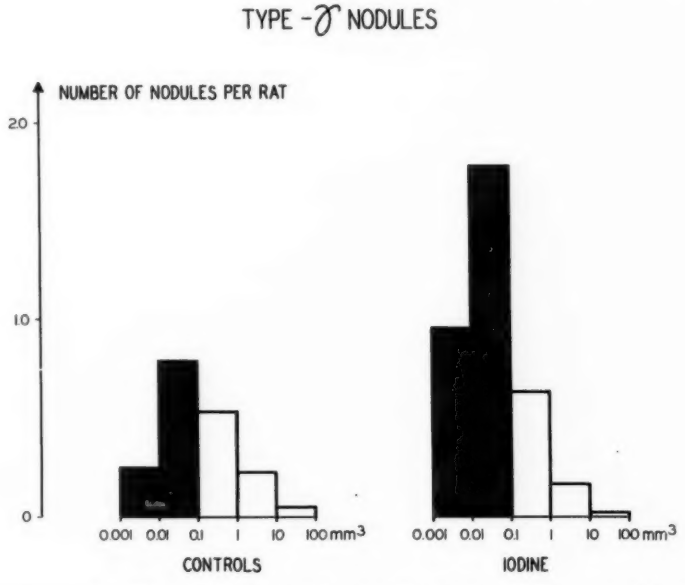
TEXT-FIGURE 6.—Effect of iodine on the size of type-γ thyroid nodules. Iodine treatment was started at time 0 and lasted 2 to 64 days. The nodules are divided into 2-size classes, namely, those smaller and those larger than 0.1 mm.³ The number of small nodules was considerably increased by iodine, while the number of large nodules was little changed. The straight lines are the best fit lines according to the least square criterion. P for regression coefficients: controls, small nodules 0.4; controls, large nodules 0.1 to 0.2; iodine, small nodules 0.001 to 0.01; iodine, large nodules 0.5.

culating thyrotrophic hormone. (2) Type-β nodules have been observed in the thyroid of animals treated with thiourea-like compounds (*11–15*), which are known to produce also a high level of thyrotrophic hormone (*16*). (3) Type-β nodules are not produced by the low-iodine diet if the rats are supplemented with iodine (*3*).

Since a high and sustained level of thyrotrophic hormone is apparently required for the onset of type-β nodules, the question arose as to whether this condition is or is not required for their maintenance and growth. The present investigation was designed to examine the fate of these nodules

when thyrotrophic hormone is brought back to a normal level. Iodine administration was expected to lower the thyrotrophic hormone level, and, in fact, it did. After 2 days of iodine treatment, the "cytological coefficient" had dropped from 20 to 15. Since this coefficient varies linearly with the logarithm of the dose of hormone (*7*), this is in fact a 50 percent decrease in the actual hormone level in blood. Although this level was still high, it was possibly not high enough to maintain small nodules, since most of these had disappeared after 2 days. Although this observation lacks the necessary statistical significance for drawing a definite conclusion, it suggests that the early phase of type-β nodule formation may require a very high level of thyrotrophic hormone, and may be reversible; that is, the nodule may be made to disappear.

When iodine treatment was extended, the level of thyrotrophic hormone in blood decreased further, and by 16 days was brought back to the normal range. Nevertheless, no disappearance of type-β nodules was observed during the period extending from 2 to 64 days; the nodules only diminished in size. The persistence and regression of the nodules confirm a similar observation made in a preliminary experiment (*2*). While it is possible that some nodules might disappear if iodine treatment were carried out for longer periods, it is unlikely that all would do so, in view of the large size maintained by some after 2 months' iodine administration. Thus, it



TEXT-FIGURE 7.—Effect of iodine on the size distribution of type-γ nodules in the animals killed after 64 days of iodine treatment. The height of the blocks indicates the number, per rat, of nodules falling into a given size range, which is indicated in $mm.^3$ on the abscissa. The number of small nodules (black blocks) was considerably increased, while that of the large nodules (white blocks) was little changed by iodine.

seems that after a possible early reversible phase of nodule formation, there is another phase characterized by the fact that the nodules do not depend on a high level of thyrotrophic hormone for their persistence, although they do depend on the hormone for growing or merely maintaining their size.

Furth and his collaborators have shown that in the course of successive transplantation of pituitary tumors the transplants go through two phases (*17*). At first, for a certain number of transplantation generations, the transplants depend on a low level of thyroid hormone, in the host, to take. During this so-called "conditioned or dependent" phase, thyroid hormone administration prevents the take of the transplant; however, if the hormone is administered after the tumor has been allowed to grow, then it does not arrest but only restrains the tumor. When transplantation is carried on further, the tumor transplant becomes eventually independent; that is, it takes, no matter how high the thyroid horomone level in the host.

The concept of tumor dependency and autonomy is derived from transplantation experiments and therefore cannot be applied to primary tumors without modifying the criteria of dependency. But in the primary type-β tumors induced in the thyroid gland by iodine deficiency, the situation may also involve more than one phase. It is possible that during an early phase the nodules are highly dependent on thyrotrophic hormone for their persistence, while later they no longer depend on this hormone for persistence but only for growth. This situation compares well with the "conditioned" phase described by Furth (*17*).

No evidence was found for the presence of fully autonomous type-β nodules, that is, nodules which did not depend on a high level of thyrotrophic hormone for their maintenance and growth. On the contrary, the diminution in cell size as well as the rare mitoses observed in type-β nodules, even in the largest, indicates that probably all nodules responded to some degree to iodine treatment.

Type-γ nodules.—Type-γ nodules are found in old nongoitrous rats (*1–3*), but their incidence is higher in the goiters induced by iodine deficiency (*3*). Thus a high level of thyrotrophic hormone is apparently not required for this nodule to develop in the thyroid, and the role of this hormone in the production of type-γ nodules by the low-iodine diet is not clear.

The present experiment shows that the decrease in blood thyrotrophic hormone to a normal level was not accompanied by a regression of type-γ nodules, but, on the contrary, by a marked increase in their number. It is unlikely that this enhanced development of nodules was a result of the low level of thyrotrophic hormone. It is also unlikely that it was due to a general growth enhancement of the thyroid gland by the higher level of thyroid hormone (*18*), since the size of the gland and of the cells lining the follicles regressed with iodine treatment.

It is probable that the higher incidence of type-γ nodules is related to the large number of light cells in the glands of the iodine-treated rats. There is a great similarity between type-γ cells and light cells. They

are both lacking the characteristics of the simple epithelial cells (polarity, terminal bars, and contact with basement membrane and with the lumen of the follicle), as well as the intracytoplasmic colloid droplets. While in some type-γ nodules the cells differ from light cells by the amount of cytoplasm, the shape, size, and stainability of nucleus, as well as the higher mitotic activity, in other nodules the cells are so similar to light cells that no clear-cut cytological difference was found. Hence the conclusion that type-γ cells probably originate from light cells (*1*).

The increase in light cells observed in the present experiment has been confirmed quantitatively; while in a normal rat the ratio of light cells to simple epithelial-cell type amounts to a few percent in rats fed the low-iodine diet for 8 months, and then given iodine for 15 days, there were 34 light cells per 100 epithelial cells (*10*).

Thus, iodine produced a different effect on the 2 thyroid-cell types: It stimulated the light cells and depressed the simple epithelial-cell type. Similarly, it stimulated type-γ nodule formation and produced a regression of type-β nodules. It is likely that the increase in light cells and the stimulation of type-γ nodule development were related to each other. The presence in the thyroid gland of numerous structures intermediate between typical type-γ nodules—and areas with unusually large numbers of light cells—suggests that the new nodules which appeared during iodine treatment may have resulted from a progressive growth of the light cells accompanied by a progressive disappearance of cells of the epithelial type.

REFERENCES

(*1*) Axelrad, A. A., and Leblond, C. P.: Induction of thyroid tumors in rats by a low iodine diet. Cancer 8: 339–367, 1955.

(*2*) Leblond, C. P., Isler, H., and Axelrad, A.: Induction of thyroid tumors by a low iodine diet. Canad. Cancer Res. Conf. 2: 248–266, 1957.

(*3*) Isler, H., Leblond, C. P., and Axelrad, A. A.: Influence of age and iodine intake on the production of thyroid tumors in the rat. J. Nat. Cancer Inst. 21: 1065–1081, 1958.

(*4*) Chaney, A. L.: Improvements in determination of iodine in blood. Indust. & Engng. Chem. (Anal. ed.) 12: 179–181, 1940.

(*5*) Remington, R. E., and Levine, H.: Studies on the relation of diet to goiter. J. Nutrition 11: 343–357, 1936.

(*6*) Barker, S. B., Humphrey, M. J., and Soley, M. H.: The clinical determination of protein-bound iodine. J. Clin. Invest. 30: 55–62, 1951.

(*7*) Del Conte, E., and Vasena, J. F. E.: Méthode simplifiée pour obtenir le coefficient cytologique thyroïdien et la détermination de la thyréotrophine. Ann. endocrin. 13: 910–915, 1952.

(*8*) Fisher, R. A., and Yates, F.: Statistical Tables, 2d ed., Oliver & Boyd, Ltd., London, 1943, p. 4.

(*9*) Paterson, D. D.: Statistical Technique in Agricultural Research. A Simple Exposition of Practice and Procedure in Biometry, 1st ed. New York and London, McGraw-Hill Book Co., Inc., 1939, p. 138.

(*10*) Stux, M., Thompson, B., Isler, H., and Leblond, C. P.: Unpublished data.

(*11*) Griesbach, W. E., Kennedy, T. H., and Purves, H. D.: Studies on experimental goitre; thyroid adenomata in rats on Brassica seed diet. Brit. J. Exper. Path. 26: 18–24, 1945.

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(*12*) KUZELL, W. C., TRIPI, H. B., GARDNER, G. M., and LAQUER, G. L.: Diffuse and nodular hyperplasia of the thyroid gland in thiouracil-treated rats. Science 107: 374–375, 1948.

(*13*) PURVES, H. D., and GRIESBACH, W. E.: Studies on experimental goitre: thyroid tumours in rats treated with thiourea. Brit. J. Exper. Path. 28: 49–53, 1947.

(*14*) SELLERS, E. A., HILL, J. M., and LEE, R. B.: Effect of iodide and thyroid on the production of tumors of the thyroid and pituitary by propylthiouracil. Endocrinology 52: 188–203, 1953.

(*15*) MORRIS, H. P.: The experimental development and metabolism of thyroid gland tumors. Advances Cancer Res. 3: 51–115, 1955.

(*16*) D'ANGELO, S. A., STEVENS, C. E., PASCHKIS, K. E., and CANTAROW, A.: Influence of cortisone on thyroid-pituitary interaction in normal and goitrous rat. Proc. Soc. Exper. Biol. & Med. 83: 181–184, 1953.

(*17*) FURTH, J.: Thyroid-pituitary tumorigenesis. J. Nat. Cancer Inst. 15: 687–69 1954.

(*18*) SELLERS, E. A., and SCHÖNBAUM, E.: Enhancement of goitrogenic action of propylthiouracil by thyroxin. Science 126: 1342–1343, 1957.

PLATE 44

Normal thyroid tissue of rats with an adequate iodine intake (fig. 1), or fed a low-iodine diet for 12 months (fig. 2), or first submitted to the same treatment as in figure 2, and then supplemented with iodine for 2 months (figs. 3 and 4). Masson's trichrome. × 570

FIGURE 1.—In rats fed an adequate amount of iodine the thyroid gland contains large follicles filled with colloid (C). Two types of cells are observed: follicular cells with dark nucleus and well-stained cytoplasm, and light cells with large vacuolated nucleus and pale cytoplasm (L).

FIGURE 2.—In iodine-deficient rats, the amount of colloid (C) is scanty, and the cells are hypertrophied. Light cells are usually not distinguishable.

FIGURES 3 AND 4.—In rats fed the low-iodine diet for 1 year, and then supplemented with iodine for 2 months, the thyroid gland contains small follicles filled with colloid (C) (fig. 3). In some areas of the gland, light cells (L) are abundant (fig. 4).

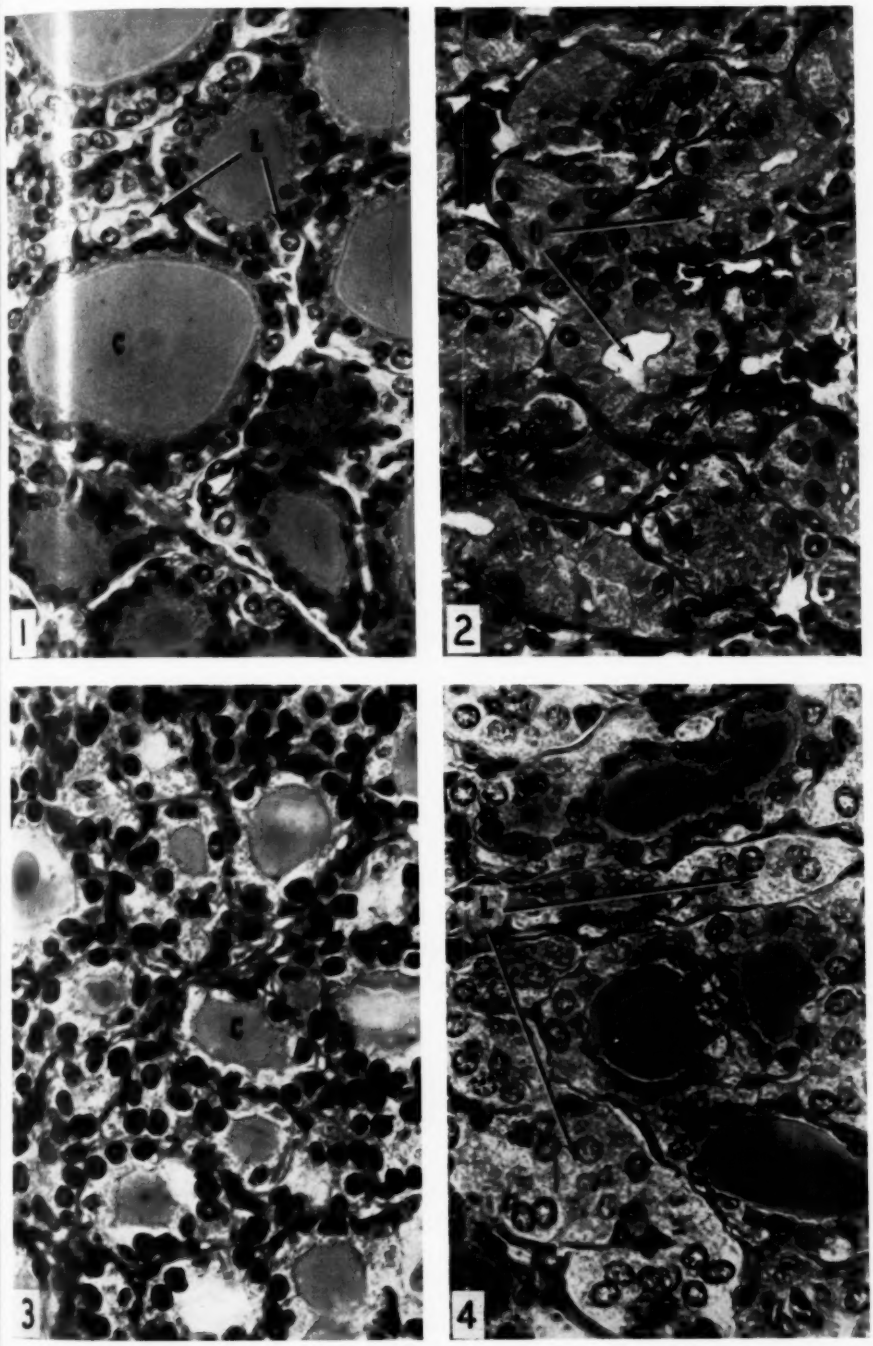
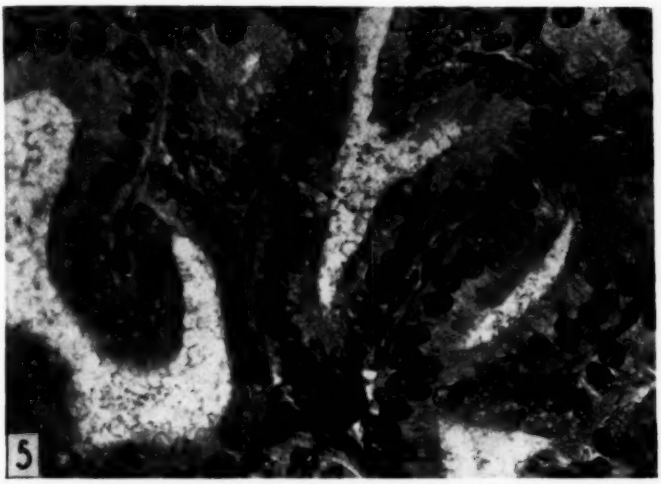

L
C
1
2
C
3
L
4

PLATE 45

Type-β nodules in thyroid glands of iodine-deficient rats (figs. 5 and 6) or of rats fed the low-iodine diet for 1 year, and then supplemented with iodine for 2 months (fig. 7). Masson's trichrome. × 570

FIGURES 5 AND 6.—Type-β nodules are follicular structures that in iodine-deficient rats may be lined by high columnar epithelium (fig. 5) or cuboidal epithelium with scanty cytoplasm (fig. 6).

FIGURE 7.—In iodine-treated rats all type-β nodules are formed of cuboidal epithelium with scanty cytoplasm.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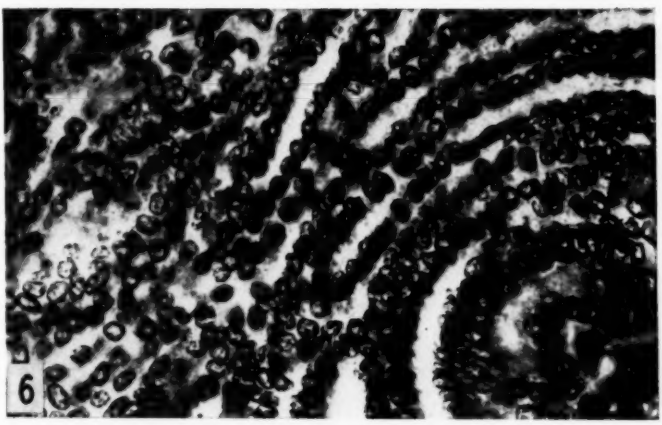
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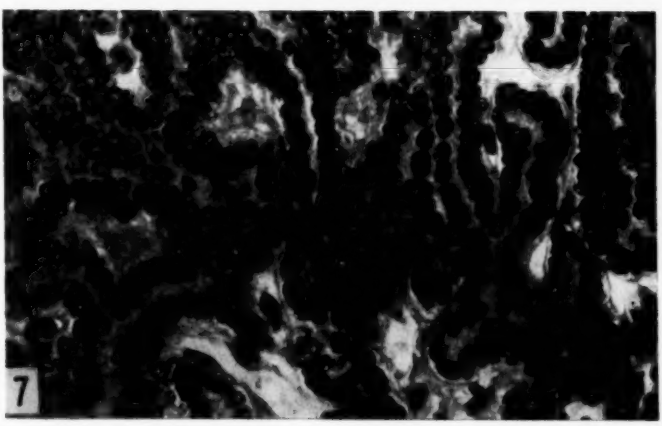
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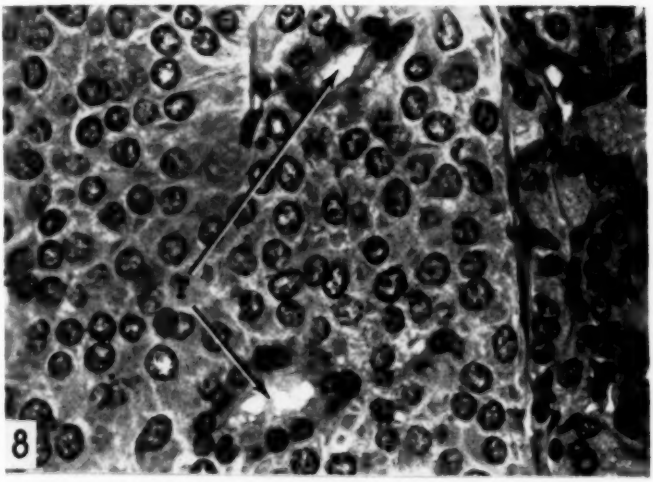
PLATE 46

Type-γ nodules in thyroid glands of iodine-deficient rats (fig. 8) and in rats fed the low-iodine diet for 1 year, and then supplemented with iodine for 2 months (fig. 9). Masson's trichrome. × 570

FIGURE 8.—Normal hyperplastic tissue at extreme *right*. The rest of the field is occupied by a solid type-γ nodule. In iodine-deficient rats these nodules contain occasional follicles (F) lined by follicular cells with darkly stained nucleus.

FIGURE 9.—In rats supplemented with iodine, type-γ nodules frequently contain numerous follicles displaying an irregular orientation of their cells (F), and clumps of follicular cells (G).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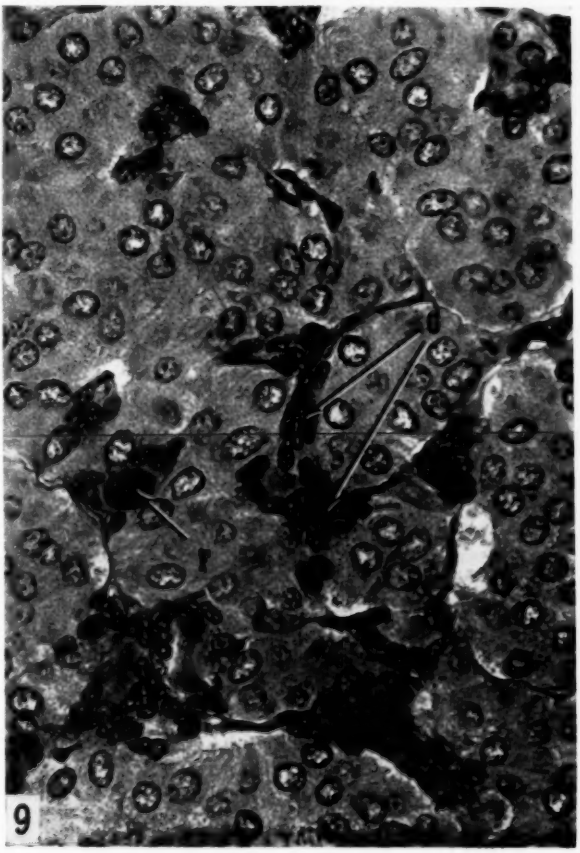
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Cytology of Rat Liver Cells During Starvation and Refeeding [1]

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SUMMARY

Seven forms of ribonucleoproteins (RNP), *viz.*, the diffuse cytoplasmic (DCR), granular cytoplasmic (GCR), perichromosomal, chromosomal, and nucleolar RNP, and the parachromatin of the nucleoplasm and the nucleolus, were demonstrated in the liver cells of normal albino rats by the toluidine blue-molybdate (TBM) method of staining. The effects of starvation and refeeding were studied on these forms of RNP as well as on the morphology of mitochondria and the cytochemistry of deoxyribonucleic acid (DNA), lipides, glycogen, protein-bound *alpha*-amino, disulfide (SS), and sulfhydryl (SH) groups, and diphosphopyridine nucleotide (DPN) diaphorase activity. Starvation caused a reduction in the size of the cell, a decrease in the size and an increase in the number of nucleoli, and swelling of the mitochondria, without alteration in the size of the nucleus. There was a decrease in DCR, GCR, and nuclear parachromatin, disappearance of nucleolar parachromatin, and little or no alteration in perichromosomal, chromosomal, and nucleolar RNP. Little change in the distribution of DNA was observed. During starvation a decrease was followed by an increase in glycogen; the amount of stainable lipide increased and ceroid appeared; the distribution of various protein-bound groups was identical with that of the DCR, but there was no diminution in intensity of staining. DPN diaphorase activity diminished during starvation. After refeeding, nucleolar RNP, nucleolar parachromatin, DCR, and GCR were quickly restored; shortly afterwards, the nuclear parachromatin increased. Chromosomal and perichromosomal RNP were unaltered. The morphological and metabolic significance of the changes is discussed.—**J. Nat. Cancer Inst. 23: 695–715, 1959.**

RECENTLY A new staining procedure which differentiates deoxyribonucleoprotein (DNP) and 7 different forms of ribonucleoproteins (RNP) has been described (*1*). The method utilizes the fact that inactivation of protein-bound amino groups by formaldehyde and nitrous acid liberates increasing numbers of nucleic acid phosphoryl groups to bind the

[1] Received for publication March 24, 1959.

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[3] We wish to thank Miss Barbara Ensfield and Mr. Roscoe H. Liles for technical assistance, and Mr. G. Gsell for photomicrography.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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cationic dye, toluidine blue. The rate at which the phosphoryl groups become available is not the same for each nucleoprotein. The amino groups of DNP are much more resistant than those of RNP, and different ribonucleoproteins also vary in susceptibility to inactivation. At a given point in the course of inactivation of amino groups, different complexes are formed between the phosphoryl groups of various nucleoproteins and dye cations. Some of these nucleic acid-dye complexes react with polymolybdates to produce metachromasia. The production of a small amount of metachromatic color at sites of strong orthochromatic staining by toluidine blue has a hypochromic effect; larger amounts of the metachromatic complex eclipse orthochromatic staining and produce intense metachromasia. Other types of bonding between nucleic acids and toluidine blue are unaffected by treatment with polymolybdates. Various nucleoproteins can therefore be distinguished by their response to deamination and subsequent coloration by toluidine blue and polymolybdate.

Undoubtedly, physical, chemical, and metabolic differences determine the response of nucleoproteins to deamination and coloration by toluidine blue and molybdate. A certain surface charge density and steric configuration of the molecule is necessary to permit the incorporation of water of dimerization to produce metachromasia (*1*, *2*). The mechanism of the toluidine blue-molybdate stain cannot be investigated in the test tube where nucleic acids are denatured, sterically altered, and isolated from their normal environment in the cell. Differences in nucleoproteins that are demonstrated by the toluidine blue-molybdate (TBM) method cannot be directly correlated with those that have been revealed by physical and chemical studies of isolated cell fractions. It should be possible, however, to obtain this knowledge indirectly by comparing alterations in the nucleoproteins shown by the TBM method with those demonstrated by other techniques. For this reason the nucleoproteins of the rat liver have been demonstrated by the TBM method, during starvation and refeeding, and an attempt has been made to correlate the results with those obtained by other means (*3–13*). A more limited study has also been included of various cell components, such as glycogen and mitochondria, which may influence the formation (*9*) or distribution of nucleoproteins (*14–16*).

MATERIAL AND METHODS

Starvation and refeeding.—Sixty-three female Osborne-Mendel rats, approximately 7 weeks old, were used. Except during starvation, the animals were maintained on Purina chow and water *ad libitum*. The rats were housed individually in separate cages. Early experiments revealed that cytoplasmic RMP still persisted in significant amounts after 7 days of starvation, and even at 1 hour after refeeding, recognizable changes in quantity and distribution of RNP had already taken place. In subsequent experiments, therefore, an attempt was made to starve animals for still longer periods and to concentrate on earlier phases of refeeding. The

TABLE 1.—Time of death of rats during starvation and refeeding

Duration of fast	Number of rats killed
0 (normal)	6
24 hours	4
48 hours	2
96 hours	2
7 days	6
9 days	1
Time of death after refeeding*	
½ hour†	3
¾ hour	2
1 hour	4
1½ hours	2
2 hours	3
3 hours	2
4 hours	1
6 hours	2
24 hours	2
48 hours	1
4 days	1
7 days	1
Total	45

*Actual time after rats were seen to eat food.
†All refed rats except one in the ½-hour group had been starved for 7 days; this rat was starved for 9 days. The animals were weighed before death and their general condition noted. The rats were killed between 9 and 10 a.m. by a blow on the back of the neck.

experiments were repeated on 4 occasions. Eighteen animals died on the 8th day of starvation. The 45 rats were studied as shown in table 1.

Cytological studies.—Small blocks were taken from the left lateral lobe of the liver and prepared for cytological studies, as indicated in table 2.

RESULTS

Condition of the Animals

The mean weight of the animals decreased from 140 ± 2.1 gm. at the beginning of the experiment to 100 ± 6.9 gm. and 80 gm. after starvation for 7 and 9 days, respectively. The animals were weak, sometimes apathetic, or even moribund. There was, however, appreciable variation in general condition; some, though weak, were more active than others.

Morphological Changes

Cytoplasm, nuclei, and nucleoli.—The liver cells became smaller during starvation, but the nuclear size was not significantly altered. The nucleoli decreased in size and became more numerous during fasting, but became larger and less numerous on refeeding.

Mitochondria.—In the normal liver, the mitochondria were fine and rod-shaped in the center of the lobule, and small, round, and granular at the periphery (fig. 1). During fasting, the mitochondria became pro-

TABLE 2.—Cytological techniques used for the study of rat liver cells

Entity demonstrated	Fixative	Duration of fixation	Temperature	Preparation (paraffin section, frozen, etc.)	Method
Nucleoproteins	Formol sublimate	1 hour	Room	Paraffin	TBM method with RNAse* and TCA† extraction controls (*1*)
DNA	Formol sublimate	1 hour	Room	Paraffin	Feulgen reaction
Protein-bound, alpha-amino, and other groups	Formol sublimate	1 hour	Room	Paraffin	Ninhydrin-Schiff (*17*). Dinitrofluorobenzene (DNFB) (*17*)
Protein-bound disulfide (SS) and sulfhydryl (SH) groups	Formol sublimate	1 hour	Room	Paraffin	Gomori's modification of Bennett's RSR (*18*)‡
Ceroid	Formol sublimate	1 hour	Room	Paraffin	Fluorescence in ultra violet light (*19*). Acid-fast stain. PAS.§ Sudan black in 70 percent alcohol. Schmorl's reaction (*20*)
Lipides	Formol sublimate	Overnight	Room	Frozen	Sudan black in 70 percent alcohol. PAS with diastase and lipide extraction control (*21*, *22*)
Glycogen	Bouin's Frozen-dried	Overnight —	Room —	Paraffin —	PAS with diastase controls
Diphosphopyridine nucleotide (DPN) diaphorase	Formol calcium	Overnight	4° C.	Frozen	Nitro-BT method (*23*, *24*)
Mitochondria	Regaud's Formol sublimate Formol calcium	12 days 1 hour Overnight	Room Room Room	Paraffin Paraffin Frozen	Iron hematoxylin (*25*) Sudan black

*RNAse = ribonuclease.
†TCA = trichloroacetic acid.
‡RSR = red sulfhydryl reagent.
§PAS = periodic acid-Schiff.

gressively plumper, larger, and rounded or oval. After 9 days, the whole cytoplasm appeared to be almost completely filled with enormously swollen, round mitochondria (fig. 2). As early as 1 hour after refeeding, elongation of mitochondria could be detected and, later, the normal morphology was gradually regained. The normal structure was attained within 2 days after refeeding.

Cytochemical Changes

Nucleoproteins.—Nucleoproteins were demonstrated by the toluidine blue, the TBM method, and by the Feulgen reaction. The specificity of the stains for RNP and DNA was checked by observing the effect of RNase digestion and extraction by trichloroacetic acid. The intensity of staining by the TBM method is not related to the amount of nucleic acids present in the cell. Assessment of the intensity of staining by toluidine blue alone at *p*H 3 after deamination in nitrous acid provided some indication of the amount of nucleic acid in the cell.

The various types of RNP of the normal liver and their staining reactions by the TBM method are summarized in table 3. The granular cytoplasmic RNP (GCR) was distinguished from the diffuse cytoplasmic form (DCR) not only by its granular distribution but by its different response to agents that inactivate amino groups. The granular form reached its maximal intensity in stage II of fixation; with deamination, it often faded completely before the metachromasia of the DCR became apparent. Furthermore, in dividing cells, the granular form disappeared during mitosis while the diffuse RNP was unaltered (*26*). In normal livers the DCR was present in coarse clumps or in a diffuse form (fig. 3). The aggregates of DCR were bigger in the central zone of the lobule than in the peripheral zone. The GCR consisted of aggregations of abundant, compactly arranged granules (fig. 4). The chromatin was surrounded by perichromosomal RNP which had the same staining reactions as the GCR. The intensity of staining of the chromatin in stage III preparations was reduced by pretreatment with RNase. The RNP component of the chromatin that was removed by the enzyme has been designated chromosomal RNP (table 3). The RNP of the nuclear parachromatin (nucleoplasm) was present in moderate amount and was disposed in a granular pattern. Minute metachromatic granules of RNP were observed in the green nucleolus in stage I of staining (fig. 5). Because of their similarity in staining to parachromatin, these have been called nucleolar parachromatin (*27*).

During starvation, even from the 1st day onward, the coarse aggregates of DCR became more homogeneously distributed (fig. 6) (table 4). On the 7th day, DCR still stained considerably, though somewhat less intensely than normal. On the 9th day of starvation, however, the DCR stained very weakly. In stage III preparations, the DCR was spread around pale, spherical structures, which, in Regaud's preparation, were shown to be mitochondria (fig. 7). The DCR diminished during starva-

†TCA = trichloroacetic acid.
‡RSR = red sulfhydryl reagent.
§PAS = periodic acid-Schiff.

TABLE 3. Staining reactions of various ribonucleoproteins (RNP) of rat liver by TBM method

Stage	Fixation or deamination	Parachromatin	Nucleolus	Nucleolar parachromatin	Perichromosomal RNP	Granular cytoplasmic RNP (GCR)	Diffuse cytoplasmic RNP (DCR)	Chromosomal RNP
I	1 hour formol sublimate room temperature	Purple ++*	Green ++	Purple ++	NR†	NR	Green ++	NR
II	1 hour formol sublimate room temperature + 3 to 4 hours formol sublimate 37° C.	Purple + or NR	Green +	NR	Purple ++	Purple ++	Green +	NR
III	1 hour formol sublimate room temperature + overnight HNO_3	NR	Purple +++	NR	NR	NR	Purple +++	Purple +‡

*+ to +++ indicates intensity of staining.
†NR = not recognizably stained.
‡Intensity of purple staining of chromatin was slightly reduced by pretreatment with RNase.

TABLE 4.—Summary of changes in nucleoli and ribonucleoproteins during starvation and refeeding*

Experimental conditions	Parachromatin of nuclear sap	Nucleolus		Nucleolar parachromatin	GCR and DCR	
		Number	Size		Intensity of staining	Distribution
Starvation	Decreased	Increased	Decreased	NR†	Diminished	More diffuse
Refeeding Early	Unchanged	Normal	Increasing but still smaller than normal	Increased	Increasing but still diminished	Clumps reforming
Late	Normal	Normal	Normal	Increased	Normal	Normal

*Perichromosomal RNP and chromosomal RNP were not detectably altered.
†NR=not recognizably stained.

tion. After 7 days of starvation, the fine, numerous, closely packed granules of normal livers were replaced by fewer, coarser, and more widely separated granules (fig. 8). At 9 days, the granules had almost entirely disappeared, and the few that were left were very fine (fig. 9). The perichromosomal and chromosomal RNP did not show any change. The parachromatin was diminished and the nucleolar parachromatin disappeared on starvation. This was not just due to an alteration in the amount of inactivation of amino groups required to stain parachromatin, since a wide range of fixation times still failed to reveal as much parachromatin as in the normal liver cell. Nucleoli still stained intensely. The changes were earlier and more marked at the periphery that at the center of the lobules.

As early as one-half hour after refeeding, changes were detected (table 4, early refeeding). The GCR formed clumps that quickly resumed their normal proportions. In a few cells it was possible to see the strands of granules of GCR stretching between nuclear membranes and the cytoplasm (fig. 10). However, a significant number of GCR granules was present in the middle and peripheral portions of the cytoplasm as well. The perichromosomal and chromosomal RNP did not show any appreciable change. The nucleolar parachromatin quickly reformed and was even more prominent in early stages of refeeding than in the normal cell (table 4, early refeeding). The intensity of staining of nucleolar parachromatin later diminished to normal at 7 days. The nuclear parachromatin increased in amount at a stage later than the nucleolar parachromatin (table 4, late refeeding). One-half to 4 hours after refeeding, nucleoli and cytoplasm were well stained with toluidine blue after deamination, occasionally even more intensely than in the normal cell. The reconstitution of RNP was evident earlier in the center of the lobule and was complete 7 days after refeeding.

DNA.—Apart from the modification of pattern caused by the larger number of smaller nucleoli in starved animals, no other significant change throughout the experiments could be detected in the amount or distribution of Feulgen-positive material. No change could be found in the nucleolar-associated chromatin at any stage (figs. 11 and 12).

Protein-bound SH and SS groups and amino groups.—Chromatin, nucleoli, and cytoplasm were stained positively. The staining by Bennett's reagent could be blocked by pretreatment with saturated mercuric chloride, which indicated that the color reaction was specific for SS and SH groups. In the cytoplasm, the site of positive reaction for SS and SH groups, ninhydrin-Schiff, and DNFB reactions closely corresponded with that of the DCR (fig. 13). The GCR was not distinguishable by these techniques. The clumped appearance of the cytoplasm of the normal liver cell was replaced by diffuse staining after starvation, but the intensity of staining was little affected (fig. 14). After refeeding, the pattern of distribution was restored to normal, almost at the same time as that of RNP.

Lipides.—The amount of sudanophilic material in the liver cells in-

creased during starvation. As noted later, PAS-positive, diastase-resistant material which could be extracted with methanol-chloroform (perhaps representing Golgi lipide) increased in amount on starvation. In unstained paraffin sections of the livers of 2 animals that survived until the 9th day of starvation and in 5 that were starved for 7 days and were in poor general condition, the cytoplasm contained minute, pale-brown, refractile granules (fig. 15). The granules showed characteristic fluorescence of ceroid in ultraviolet light, and were sudanophilic and PAS-positive; they gave a negative Schmorl reaction and were only weakly acid fast. The properties of this pigment therefore conform to those of ceroid (*19*). The sudanophilia of the mitochondria was greatly increased during starvation, which suggested that mitochondrial lipides might have been unmasked by starvation.

Glycogen.—In normal animals, diastase-sensitive, PAS-positive material was present as cytoplasmic clumps in material fixed in Bouin's fluid. It was more diffusely distributed in frozen-dried material. The amount of demonstrable glycogen decreased after 24 hours of starvation. The intensity of staining increased from 48 hours onward and, at 7 days of starvation, glycogen was very strongly stained (fig. 16). The amount of glycogen demonstrated by the PAS method increased further after refeeding. The distribution in fixed and frozen-dried material was not directly related to that of RNP. Glycogen could be demonstrated in places where RNP was present as well as in areas where RNP was absent (figs. 17 and 18).

Diastase-resistant PAS-positive material.—An extremely small amount of diastase-resistant PAS-positive material was present in normal livers. The intensity of staining of this component was reduced but not completely abolished by extraction of lipides by chloroform-methanol. Thus a part of this diastase, insensitive PAS-positive material was lipide and could not be ceroid, since it differed in distribution. Ceroid was more diffusely distributed in the cell; this material was more circumscribed, probably in the area corresponding to the Golgi region. Chemically this substance could comprise phospholipide, glycolipide, and/or glycoprotein. The intensity of staining of this area by protein stains was not distinguishable from that of the rest of the cytoplasm.

The PAS-positive protein (and/or lipide) in the Golgi zone increased progressively during starvation and decreased slowly during refeeding.

DPN-diaphorase.—As a supplement to the morphological study of mitochondria, the DPN-diaphorase activity was studied as an index of the physiological activity of mitochondria. In normal livers there was a more marked reduction of Nitro-BT in the central zone in comparison with the periphery of the lobule (fig. 19). During starvation, the general intensity of Nitro-BT reduction diminished, though less so in the center of the lobules (fig. 20). On refeeding, even after 1 hour, the diaphorase activity increased. This was more marked in the center of the lobules.

Whereas the changes observed during starvation and refeeding were generally as described, there were appreciable individual differences in

animals in the same stages of the experiment. Thus, after 7 days of starvation, RNP was only slightly altered in some animals, while in others the changes were very conspicuous.

DISCUSSION

The functional significance of the results of the present study may be better understood by relating these observations to those derived by other techniques. The range of biological variation under normal and abnormal conditions should be clearly appreciated. In some studies, this variability has been emphasized (*9*) or dealt with by using a large number of animals, with statistical analysis of the data (*3*). In the present study, marked variations were found in the cytological appearance at each stage of the experiment. There are known to be histochemical variations in different parts of the liver itself (*28, 29*). These factors must be taken into consideration in the interpretation of electron-microscope studies in which small pieces of tissues derived from a small number of animals have been examined.

The present observations confirm the reduction in size of liver cells and nucleoli during starvation, described by others (*3, 8*). The nuclear size, which has been said to be decreased (*7*), increased (*11*), or unaffected (*5*), was found unchanged in the experiments reported here. Differences in strain, age, sex, and antecedent diet of the animals in these studies may be responsible for the variable results. In agreement with the studies of Lagerstadt (*3*), more nucleoli per nucleus were found after starvation; on the other hand, no difference in the distribution of nucleoli within the nucleus could be noted (*30*).

Elucidation of the metabolism of RNP has been hindered by the lack of specificity of the staining procedures (*31*) or by the poor correlation between the content of RNP and basophilia (*32*) or the intensity of ultraviolet absorption by tissues (*33–35*). The TBM method used in the present study is also not strictly quantitative, but toluidine-blue staining at low *p*H after overnight deamination of tissues gives a rough idea of the content of ribonucleic acid. By this procedure the majority of RNP phosphoryl groups are made available for binding with the dye, since competition between the dye and the protein-bound amino groups is virtually eliminated. The complete disappearance of RNP from the cytoplasm of liver cells, which has been inferred from the absence of cytoplasmic basophilia after starvation (*3*), has not been confirmed by biochemical (*5, 7, 10*), cytological (*16*), and electron-microscopic (*6, 9, 36*) studies, which indicate only a diminution of RNP. The study reported here is in conformity with the latter findings. Replacement of big clumps and granular aggregates by a homogeneously spread mass of RNP could be interpreted as disaggregation of the ergastoplasm, which is noted in electron micrographs (*6*). This state of diffuse dispersion would lead to a decrease in basophilia (*6, 12, 37*).

At present it is difficult to correlate the granular cytoplasmic and diffuse forms of RNP revealed by the TBM method with the cell fractions used in biochemical studies and the studies described by electron microscopists. The size of the cytoplasmic granules revealed by the TBM method is much greater than that of the sites of dye-binding, because staining is produced by the formation of a metachromatic dye-molybdate precipitate (*1*). The cytoplasmic granules, however, could not be Palade granules, which are only 100 to 150 Å in diameter. It is equally difficult to correlate the diffuse cytoplasmic RNP either with the endoplasmic reticulum or with the supernatant or cell-sap fraction prepared by differential centrifugation. The GCR does, however, change in distribution during starvation and appears to diminish in amount in the same manner as the ergastoplasm; this in turn links the granules well with the microsomal fraction, which contains the elements of the endoplasmic reticulum and Palade granules (*38*). The diffusely stained RNP occupies the same general areas as the granular form, both in the normal liver cell and after starvation. On the basis of binding of toluidine blue after deamination, the RNP content of this component appears to diminish during starvation. It is possible, therefore, that the diffusely staining RNP may correspond to the cell sap (*39*) or "nonpellet-RNP" (*13*), which is also reduced during starvation.

In the nucleus, RNP is present in the nucleolus, parachromatin (or nucleoplasm) and in and around the chromosomes (*1*, *11*, *12*, *40*). In the present study the chromosomal and perichromosomal RNP were unaltered, while the amount of demonstrable parachromatin decreased during fasting. The simultaneous reappearance of the nucleolar parachromatin and the cytoplasmic granular RNP before the reappearance of the rest of the nuclear parachromatin suggests that the GCR could not be directly derived from the parachromatin. The amount of nucleolar parachromatin is greater than normal immediately after refeeding and gradually decreases as the rest of the parachromatin is restored to normal. Some of the nuclear parachromatin may therefore be formed in the nucleolus. Although the perichromosomal RNP was unaltered throughout the experiment, occasional connections between this form and the regenerating GCR were seen in the early stages of refeeding. This in itself does not prove the direct passage of RNP from the nucleus. The staining properties of the perichromosomal and the GCR are similar (*1*), and it is possible that rapid extrusion of RNP from the nucleus may contribute to the reconstitution of the GCR. The demonstration of nuclear pores (*6*, *41*) provides a basis for nucleocytoplasmic interchange of nucleoproteins, but biochemical differences between the nuclear and cytoplasmic RNP (*42*, *43*) suggest that synthesis of all RNP by the nucleus is unlikely. Nucleolar and perichromosomal RNP, which did not change appreciably during starvation, may be very different metabolically from other forms.

The prominence of nucleolar parachromatin in the early stages of refeeding, when both RNP and protein synthesis and not mere turnover

was taking place in the cell, is of interest. A similar increase in this material has been noted in the nucleoli of cells infected with Newcastle-disease virus (NDV), in which RNP of viral origin is being synthesized (*44*). The nucleolar parachromatin also disappears during prophase and is absent during metaphase and anaphase (*26*) when the RNP synthesis is greatly diminished (*45*).

Liver DNA has been established as the least variable of the cellular characteristics (*5*). Contrary to previous observations (*3*, *4*), no significant alteration in chromatin pattern was noted in the present study. It is felt that no special pattern of chromatin may be associated with different states of RNP metabolism in the liver during starvation and refeeding.

The similarity between the distribution of the protein-bound groups revealed by the ninhydrin-Schiff, DNFB, and Bennett procedures and that of RNP in the cell suggests that these groups may be components of the protein fraction of the RNP. There is biochemical and electron-microscopic-cytochemical evidence (*11*) for the association of SH groups with RNP macromolecules. The protein-bound groups stained by ninhydrin-Schiff and DNFB were unaltered during the decrease in RNP. The known decrease in protein content of liver cell during starvation may therefore be largely the result of diminished cell size rather than decreased protein concentration.

It has been claimed that glycogen will displace the RNP of the liver cell and thus determine distribution (*46*). In the present study, glycogen was clearly demonstrated in the same areas as the RNP, as well as in other parts of the cell. The results suggest that glycogen does not determine the distribution of RNP in the cell. Cell fractionation (*47*, *48*) and cytological (*6*) studies confirm these observations and indicate that glycogen is diffusely distributed in the cell.

During starvation, RNP was distributed as a fine network surrounding the swollen forms of mitochondria. Since mitochondria are very rich in important energy-yielding enzymes, it is possible that these enzymes may participate directly or indirectly in cytoplasmic synthesis of RNP. However, the swollen mitochondria during starvation occupy almost all the cytoplasmic space, so that whatever RNP still persists must surround them for purely physical reasons. This close association may not, therefore, necessarily indicate any metabolic relationship, as has been suggested by Bernhard *et al.* (*9*).

A decrease in enzymes (*10*, *49*), which is followed by an increase of glycogen (*5*) and an increase of lipides (*5*) on starvation, has been noted by previous authors as well as in the present study. General cytoplasmic loss may account for enzyme loss (*10*, *49*), and other little-known metabolic processes may account for changes in glycogen and lipides. However, these findings contribute only indirectly toward an understanding of the state of RNP metabolism in the liver.

REFERENCES

(*1*) Love, R., and Liles, R. H.: Differentiation of nucleoproteins by variable deamination and staining with toluidine blue and molybdate. J. Histochem. 7: 164–181, 1959.

(*2*) Bergeron, J. A., and Singer, M.: Metachromasy; an experimental and theoretical reevaluation. J. Biophys. & Biochem. Cytol. 4: 433–457, 1958.

(*3*) Lagerstedt, S.: Cytological studies on the protein metabolism of the liver in rat. Acta anat. Supp. 9, 1949.

(*4*) ———: Hepatic histochemistry. *In* Liver Injury (Hoffbauer, F. W., ed.). New York, Josiah Macy, Jr., Foundation, 11th Conference, 1952, pp. 216–265.

(*5*) Harrison, M. F.: Effect of starvation on the composition of the liver cell. Biochem. J. 55: 204–211, 1953.

(*6*) Fawcett, D. W.: Observations on the cytology and electron microscopy of hepatic cells. J. Nat. Cancer Inst. 15: 1475–1503, 1955.

(*7*) Laird, A. K., Barton, A. D., and Nygaard, O.: Synthesis of protein and ribonucleic acid in rat liver during refeeding after starvation. Exper. Cell Res. 9: 523–540, 1955.

(*8*) Stenram, U.: Nucleolar size in the liver of rats fed on high and nonprotein diets after starvation. Acta anat. 26: 352–361, 1956.

(*9*) Bernhard, W., and Rouiller, C.: Close topographical relationship between mitochondria and ergastoplasm of liver cells in a definitive phase of cellular activity. J. Biophys. & Biochem. Cytol. 2: Supp. 4, 73–78, 1956.

(*10*) Allard, C., Lamirande, G. D., and Cantero, A.: Behavior of enzymes in liver of starved rats. Exper. Cell Res. 13: 69–77, 1957.

(*11*) Bahr, G. F.: Changes in liver cell elements during stimulated protein svnthesis. Acta. Radiol. Supp. 147, 1957.

(*12*) Finck, H.: An electron microscopic study of the basophile substances of frozen-dried rat liver. J. Biophys. & Biochem. Cytol. 4: 291–300, 1958.

(*13*) Petermann, M. L., and Hamilton, M. G.: The influence of age, sex, pregnancy, starvation and other factors on the cytoplasmic ribonucleoproteins of rat liver. J. Biophys. & Biochem. Cytol. 4: 771–776, 1958.

(*14*) Deane, H. W.: The basophilic bodies in hepatic cell. Am. J. Anat. 78: 227–244, 1946.

(*15*) Yokoyama, H. O., Tsuboi, K. K., Wilson, M. E., and Stowell, R. E.: Histochemical studies on regenerating mouse liver. Lab. Invest. 2: 91–108, 1953.

(*16*) Stenram, U.: Cytological studies on the formation of the basophilic inclusions in the liver cell cytoplasm of rat. Acta anat. 21: 386–390, 1954.

(*17*) Burstone, M. S.: An evaluation of histochemical methods for protein groups. J. Histochem. 3: 32–49, 1955.

(*18*) Gomori, G.: Histochemical methods for protein-bound sulphydryl and disulphide groups. Quart. J. Micro. Sc. 97: 1–9, 1956.

(*19*) Hamperl, H.: Die Fluorescenzmikroskopie meschlicher Gewebe. Virchows Arch. 292: 1–51, 1934.

(*20*) Pearse, A. G. E.: Histochemistry, Theoretical and Applied. Boston, Little, Brown & Co., 1954, 530 pp.

(*21*) McManus, J. F. A.: Histological and histochemical uses of periodic acid. Stain Technol. 23: 99–108, 1948.

(*22*) Lillie, R. D.: Histopathologic Technic and Practical Histochemistry. New York, The Blakiston Co., Inc., 1954, pp. 501.

(*23*) Nachlas, M. M., Walker, D. G., and Seligman, A. M.: A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. J. Biophys. & Biochem. Cytol. 4: 29–38, 1958.

(*24*) Novikoff, A. B., and Masek, B.: Survival of lactic dehydrogenase and DPNH-diaphorase activity after formol calcium fixation. J. Histochem. 6: 217, 1958.

(25) REGAUD, C.: Études sur la structure des tubes séminifères et sur la spermatogénèse chez les mammifères. Arch. d'anat. micr. 11: 291–431, 1909–1910.

(26) LOVE, R.: Distribution of ribonucleic acid in tumour cells during mitosis. Nature, London 180: 1338–1339, 1957.

(27) LOVE, R., and BHARADWAJ, T. P.: Two types of ribonucleoproteins in the nucleoli of mammalian cells. Nature, London 183: 1453–1454, 1959.

(28) BOGOCH, A., CASSELMAN, W. G. B., and BOCKUS, H. L.: Variations in the histochemical findings in liver tissue obtained by biopsy. Am. J. M. Sc. 229: 273–279, 1955.

(29) BHARADWAJ, T. P., and ANTIA, F. P.: The sample value of liver biopsy. Ind. J. M. Sc. 12: 812–813, 1958.

(30) JONSEN, M. T., and STONIG, W. F.: Waarnemingen bij lever nucleoli. Nederl. tijdschr. geneesk. 102: 3, 1955.

(31) STENRAM, U.: The specificity of the gallocyanin-chromalum stain for nucleic acids as studied by the ribonuclease technique. Exper. Cell Res. 4: 383–389, 1953.

(32) FARBER, E., WESER, D. K., SZANTO, P. B., and POPPER, H.: Correlation between cytoplasmic basophilia and the nucleic acid content of the liver. A.M.A. Arch. Path. 51: 399–408, 1951.

(33) CASPERSSON, T.: Über den chemischen Aufbau der Strukturen des Zellkernes. Skandinav. Arch. f. Physiol. 73: supp. 8, 1–151, 1936.

(34) COMMONER, B.: On the interpretation of the absorption of ultraviolet light by cellular nucleic acids. Science 110: 31–40, 1949.

(35) GORDON, M. W., and NURNBERGER, J. I.: Acute cold-induced changes in cellular protein and nucleic acids in rat liver and brain. A description and critique of biochemical and cytochemical studies. Exper. Cell Res. 8: 279–304, 1953.

(36) BERNHARD, W., HAGUENAU, F., GAUTIER, A., and OBERLING, CH.: La structure submicroscopique des éléments basophiles cytoplasmiques dans la foie, le pancréas et les glandes salivares. Ztschr. f. Zellforsch. u. mikr. Anat. 37: 281–308, 1952.

(37) MIRSKY, A. E., ALLFREY, V. G., and DALY, M. M.: The uptake of N15-labelled glycine by liver proteins. J. Histochem. 2: 376–377, 1954.

(38) PALADE, G. E., and SIEKEVITZ, P.: Liver microsomes. An integrated morphological and biochemical study. J. Biophys. & Biochem. Cytol. 2: 171–200, 1956.

(39) DAVIDSON, J. N.: Cytological Aspects of Nucleic Acids. *In* Biochemical Society Symposia No. 14 (Crook, E. M., ed.). Cambridge, Cambridge Univ. Press, 1957, p. 27.

(40) BRACHET, J.: La localisation des acides pentosenucléiques dans les tissus animaux et les oeufs d'Amphibiens en voie de developpement. Arch. de Biol. 53: 207–257, 1942.

(41) POLLISTER, A. W., GETTNER, M., and WARD, R.: Nucleocytoplasmic interchange in oocytes. Science 120: 789, 1954.

(42) ELSON, D., and CHARGAFF, E.: Regularities in the composition of pentose nucleic acids. Nature, London 173: 1037–1038, 1954.

(43) SMELLIE, R. M. S.: The metabolism of nucleic acids. *In* The Nucleic Acids (Chargaff, E., and Davidson, J. N., eds.). New York, Academic Press, Inc., 1955, vol. 2, pp. 393–434.

(44) LOVE, R.: Cytopathology of virus-infected tumor cells. Ann. New York Acad. Sc. 81: 101–117, 1959.

(45) BRACHET, J.: Les interactions nucléocytoplasmiques dans la cellule au repos et pendant la mitose. Rev. franç. étud. clin. et biol. II, 551–556, 1957.

(46) STENRAM, U.: Basophilic inclusions and rods in the liver cell cytoplasm of rat. A review. Acta Histochem. 5: 156–166, 1958.

(47) LAZAROW, A.: Particulate glycogen; submicroscopic component of guinea pig liver cell; its significance in glycogen storage and regulation of blood sugar. Anat. Rec. 84: 31–50, 1942.

(48) SACKS, J., JOHNSTON, P. M., MORTON, J. H., and HARVEY, J. A. N.: The intracellular distribution of liver glycogen. Exper. Cell Res. 12: 537–545, 1957.

(49) MILLER, L. L.: Changes in rat liver enzyme activity with acute inanition; relation of loss of enzyme activity to liver protein loss. J. Biol. Chem. 172: 113–121, 1948.

PLATES

PLATE 47

FIGURE 1.—Normal rat liver stained with Regaud's chrome-iron-hematoxylin for mitochondria. *Note* that mitochondria are fine, rod-shaped, and granular. × 1,180

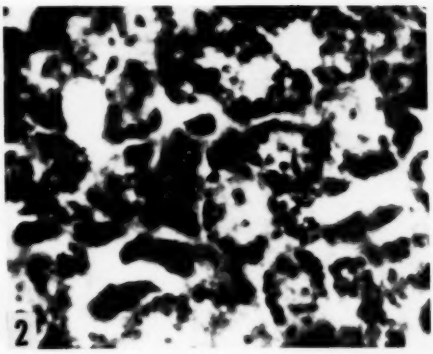
FIGURE 2.—Section from liver of rat starved 9 days, stained with Regaud's chrome-iron-hematoxylin for mitochondria. Mitochondria are swollen, round, and occupy most of the cytoplasmic space. × 1,180

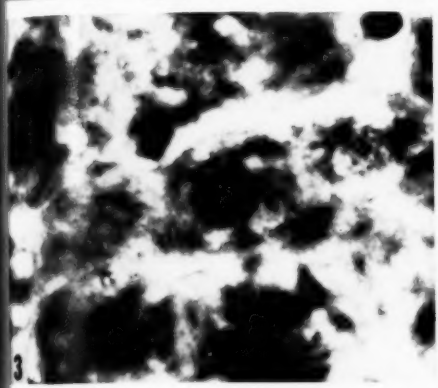
FIGURE 3.—Section from liver of normal rat stained by TBM method (Stage III). *Note* clumps as well as diffuse distribution of RNP (DCR). × 1,180

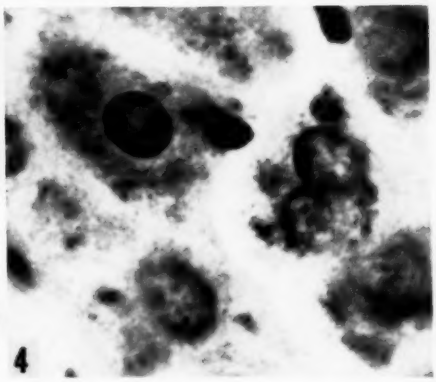
FIGURE 4.—Section from liver of normal rat stained by TBM method (Stage II). *Note* metachromatic (dark) staining of RNP around chromosomes (perichromosomal RNP) and of cytoplasmic granules(GCR), which are compactly arranged in clumps. × 1,400

FIGURE 5.—Section from liver of normal rat stained by TBM method (Stage I). *Note* faintly staining nuclear parachromatin and metachromatic nucleolar parachromatin in the form of fine granules (*arrow*). × 1,180

FIGURE 6.—Section from liver of rat starved 7 days, stained by TBM method (Stage III). *Note* absence of clumped forms of DCR. × 1,180







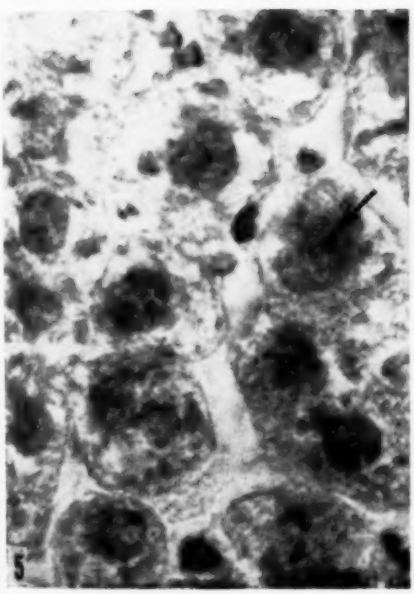


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PLATE 48

FIGURE 7.—Section from liver of rat starved for 9 days, stained by TBM method (Stage III). Cytoplasm is very pale and DCR is arranged around pale, spherical structures (mitochondria). (*Cf.* fig. 2.) × 1,180

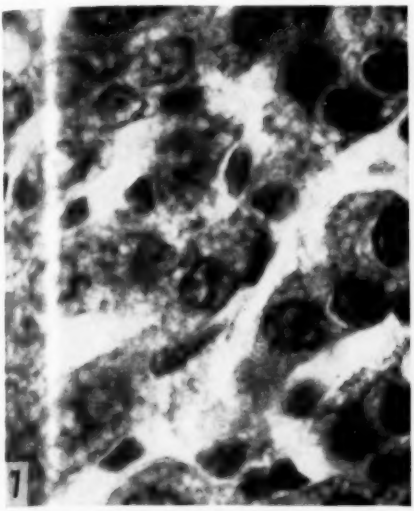
FIGURE 8.—Section from liver of rat starved for 7 days, stained by TBM method (Stage II). Granules in cytoplasm are fewer and more widely separated. Perichromosomal RNP stains intensely. × 1,180

FIGURE 9.—Section from liver of rat starved for 9 days, stained by TBM method (Stage II). Granules (GCR) have almost entirely disappeared. Perichromosomal RNP stains intensely. × 1,180

FIGURE 10.—Section from liver of rat, half an hour after refeeding, stained by TBM method (Stage II). *Note* trail of granules of GCR extending from nuclear membrane into cytoplasm (*arrows*). × 1,180

FIGURE 11.—Section of liver from normal rat stained by the Feulgen reaction. *Arrow* indicates nucleolar-associated chromatin. × 1,180

FIGURE 12.—Section from liver of rat starved for 9 days, stained by the Feulgen reaction. No decrease in nucleolar-associated chromatin (*arrows*). × 1,180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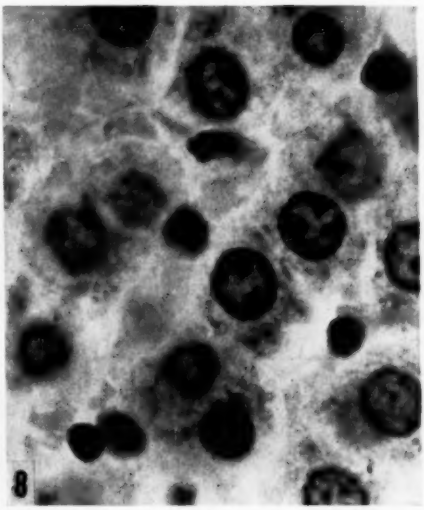
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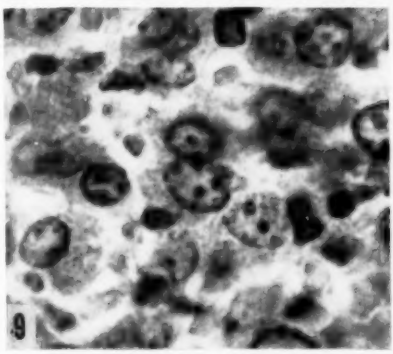
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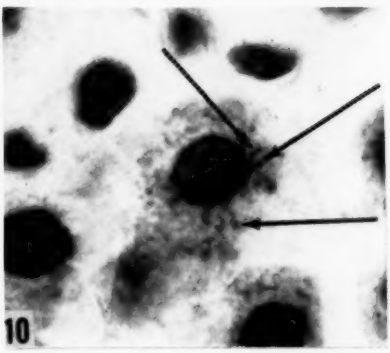
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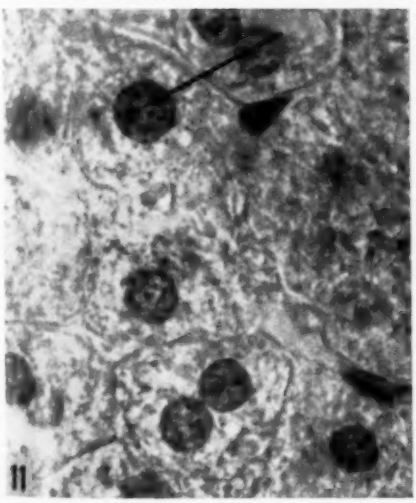
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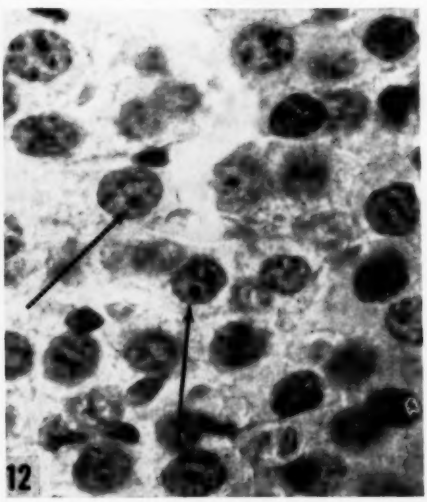
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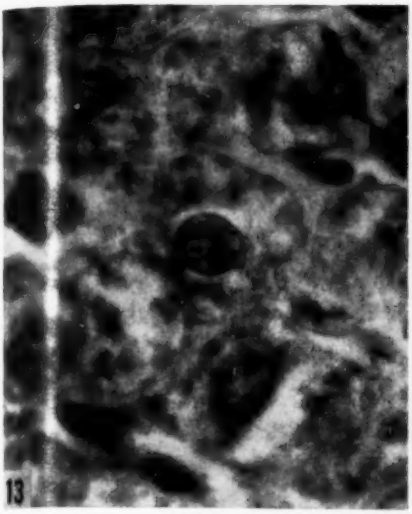
PLATE 49

FIGURE 13.—Section from liver of normal rat stained for SS and SH groups. Distribution of stainable groups is similar to that of the DCR (fig. 3). × 1,180

FIGURE 14.—Section from liver of rat starved for 7 days, stained for SS and SH groups. *Note* homogeneous distribution without much diminution in intensity of staining (*cf.* figs. 6 and 13). × 1,180

FIGURE 15.—Unstained section from liver of rat starved for 9 days. Numerous fine granules of ceroid are distributed diffusely in cytoplasm. × 1,180

FIGURE 16.—PAS-stained section from liver of rat starved for 7 days. *Note* large amount of stainable glycogen in the cell. × 1,180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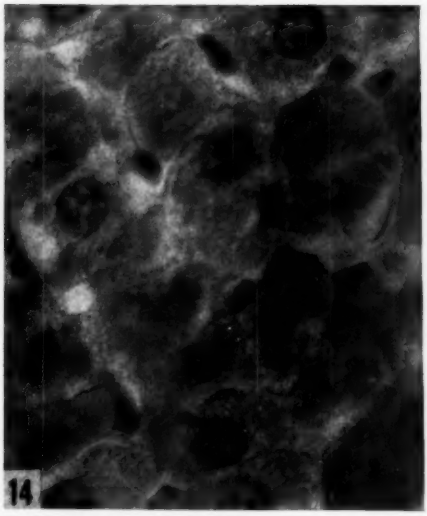
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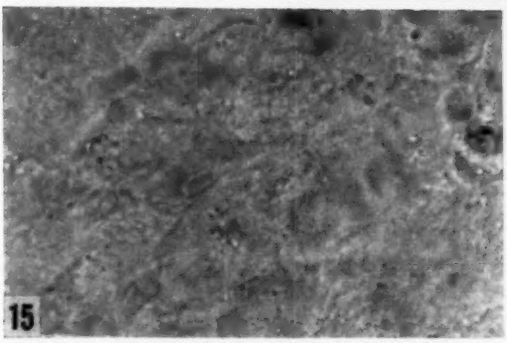
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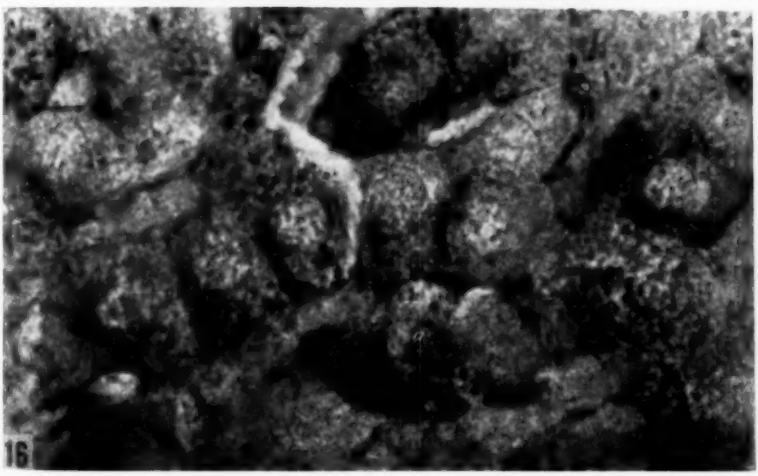
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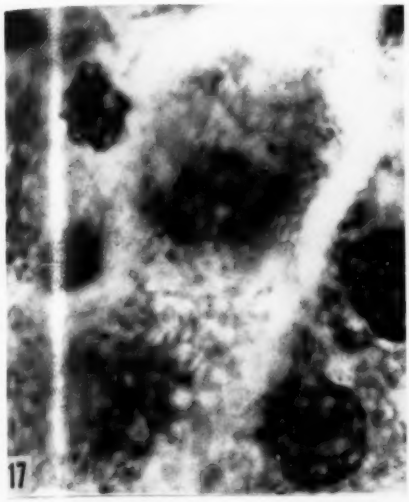
PLATE 50

FIGURE 17.—Frozen-dried section of rat liver stained by TBM method (Stage II). × 1,400

FIGURE 18.—Same section as figure 17, decolorized in 50 percent alcohol and restained by periodic acid-Schiff. *Note* that RNP stains both in areas occupied by glycogen as well as in those unoccupied. × 1,400

FIGURE 19.—Section of liver from normal rat stained for DPN-diaphorase activity. *Note* that reduction of Nitro-BT is most intense in center of lobule (*bottom*). × 1,180

FIGURE 20.—Section of liver from rat starved for 7 days, stained by DPN-diaphorase activity. There is slight diminution in reduction of Nitro-BT. Activity of enzyme is little affected in center of lobule. × 1,180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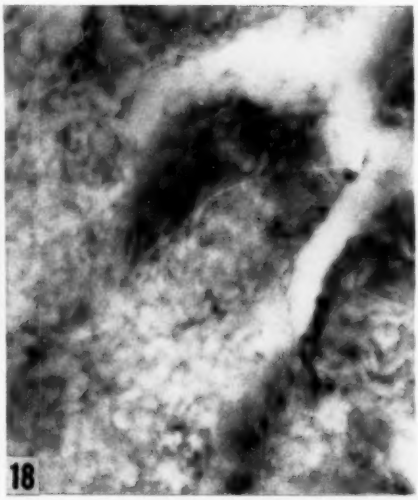
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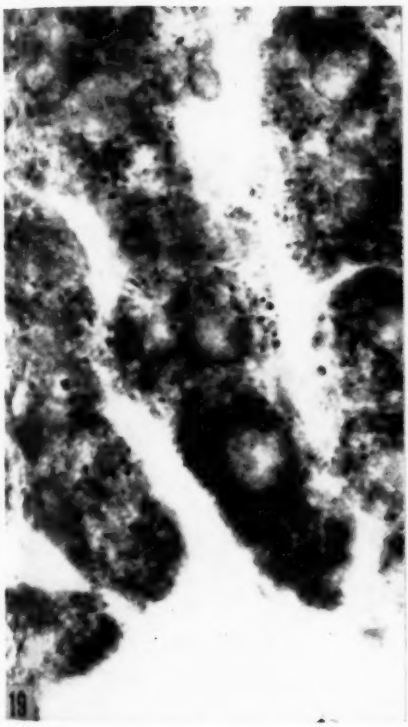
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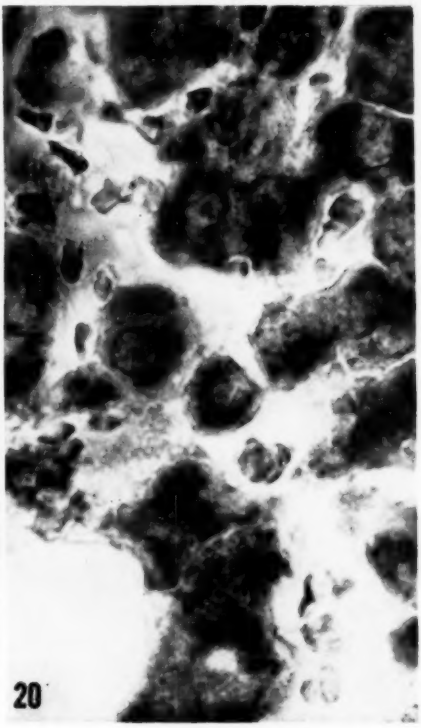
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Studies of Parotid-Tumor Agent in Cultures of Leukemic Tissues of Mice [1]

CLYDE J. DAWE, LLOYD W. LAW, *and* THELMA B. DUNN,[2] *National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Attempts to detect a presumptive leukemia-inducing or leukemia-accelerating agent in leukemic tissues of the mouse in short-term cultures have been unsuccessful, since no agent was found in a variety of leukemias, including 3 from the induced passage A series of Gross. However, parotid-tumor agent (PTA) was isolated from 2 of the 3 leukemias from the Gross series. One of the positive lines subsequently converted to negative during serial transplantation. This added to the evidence that association of leukemias with PTA is largely coincidental. Continuous cultures (P388 D_1) derived from a DBA/2 lymphoma initially gave no evidence of carrying PTA, but were susceptible to infection with PTA. Ten serial passages of PTA through P388 D_1 were completed without evident decline of tumor-inducing activity. Thymic epithelial tumors and a variety of other tumors and lesions associated only rarely with the agent before passage through P388 D_1 cultures were observed more frequently after such passage. The tumors associated with PTA form a histologically distinctive group. "Carrier" cultures of P388 D_1 infected with PTA were maintained continuously for 9 months in high-serum medium. Continued presence of the agent was demonstrated by persistent tumor-inducing activity of these cultures. No completely resistant strain of mouse was found among 12 tested with PTA. Mice up to 14 days of age were found susceptible to induction of parotid tumors by PTA of high activity. Resistance of certain entire litters to PTA appeared to be correlated with maternal immunity. Tissue-culture preparations of PTA induced tumors in all litters and in nearly 100 percent of mice born of uninfected mothers.—J. Nat. Cancer Inst. 23: 717–797, 1959.

IN A previous report Law, Dunn, and Boyle (*1*) gave detailed data on the occurrence and the histology of tumors of the parotid gland in 3 substrains and 2 F_1 crosses of C3H mice that received, shortly after birth, extracts and filtrates of leukemic tissues principally from AKR and C58 mice. Their observations in general confirmed the original reports of

[1] Received for publication March 29, 1959.

[2] We are indebted to Drs. W. P. Rowe and I. Brodsky for the determinations of hemagglutinins and of hemagglutination-inhibiting and complement-fixing antibodies used in this study. We are also grateful to Mr. Britton Smith for technical assistance in the animal work; to Mr. Willie D. Morgan for technical assistance in tissue culture; and to Mr. Gebhard Gsell for the photographs.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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Gross (*2*) and Stewart (*3, 4*), which described the induction of salivary-gland tumors by cell-free extracts of leukemic tissue. Their procedures, however, did not produce an increase in leukemia such as Gross described (*5, 6*). Dulaney (*7*), Woolley *et al.* (*8*), Latarjet *et al.* (*9, 10*), Rogel and Rudali (*11*), and Buffett *et al.* (*12*) also have reported the induction of salivary-gland tumors by cell-free extracts of leukemic tissue. Besides salivary-gland tumor induction, evidence of leukemia-inducing or leukemia-accelerating effect of certain extracts has also been described by Stewart (*3, 13*), Woolley and Small (*14*), Dulaney *et al.* (*15*), Rudali *et al.* (*16*), and Hays *et al.* (*17*), all of whom used tissues from AKR mice exposed to materials originating from Gross's laboratory.

Seeking a more effective method of demonstrating a presumptive leukemia-inducing or leukemia-accelerating agent, and attempting at the same time to establish continuous cultures of certain murine leukemias, we considered the possibility that a leukemogenic agent might be released in greater abundance or higher potency under the conditions of culture than by direct extraction of leukemic tissues. A rationale for this procedure could be found in either a virus theory or a chemical theory, *e.g.*, concept of a nucleic acid influence, as to the nature of the presumptive agent. If the agent were a virus, it might be unmasked in short-term cultures, after the manner of adenovirus unmasking (*18*). If it were a nucleic acid or other chemical compound of less complexity than a complete virus, it might be released from the leukemic-tissue explants in the course of the cell death and breakdown that are prominent in early cultivation of many types of leukemia.

As an exploratory procedure, therefore, the medium removed from cultures of leukemic tissues at feeding times was filtered and inoculated into newborn mice. It soon appeared that cultures of a lymphocytic leukemia from a C3H/BiGs mouse,[4] provided by Dr. Ludwik Gross, yielded an activity higher than that of direct tissue extracts of the same leukemia, so far as induction of salivary-gland tumors was concerned. After this observation, several leukemic and nonleukemic tissues from different mouse strains were similarly tested, and the results are described later. From none of the materials used was any significant amount of leukemia-inducing or leukemia-accelerating activity obtained.

Among the materials tested was the continuous tissue-culture cell strain P388 D_1, isolated from a lymphoid neoplasm of a DBA/2 mouse (*19*). Although no evidence was found for either a leukemia agent or parotid-tumor agent (PTA) in this continuous cell strain, Rabson and Legallais (*20*) demonstrated that P388 D_1 could propagate a strain of PTA isolated by us, as well as an apparently very similar strain (SE polyoma virus) described in mouse-embryo tissue cultures by Eddy, Stewart, and Berkeley (*21*). Another method for study of a possible relationship between leukemias of mice and PTA was thus made available. We include in this report our observations on the serial passage of agent in

[4] Our designation of the Gross subline of mice obtained from Bittner's subline C3Hf/Bi. Hereafter in the text this subline is designated as C3H/Gs.

P388 D_1 cultures, and the establishment of a "carrier" strain of P388 D_1 cells infected with PTA.

MATERIALS AND METHODS

I. Mice

All mice reported here were from the inbred strains designated in the tables, or were F_1 hybrids of these strains as specified. They were bred in the colonies of one of us (L. W. L.) and were fed a diet of Derwood pellets with water *ad libitum*.

The test animals received a single injection of test material, at less than 12 hours of age in most instances. Relatively few received injections at more than 24 hours, and those that received injections at more than 48 hours of age are specifically indicated in the tables. Litters were kept intact, each housed in an individual cage with the mother, until weaned. They were examined daily until weaned, and twice a week thereafter, for the appearance of tumors or signs of illness. After weaning, an attempt was made to cage litters individually, but when more than 8 mice were weaned in a single litter, the litter was divided. Two or more litters were placed in the same cage when there were only 2 or 3 per litter, to have all cages contain 7 to 8 mice. Most animals were killed at 12 to 15 months of age if tumors did not appear. It had been observed in the early part of this work that few neoplasms of types related to the agent appeared in the age range of 9 to 15 months.

For the most part, recipients of extracts or filtrates were mice of the C3Hf/BiLw [5] substrain or of the hybrid combination (C3H × AKR)F_1 obtained by matings of female C3Hf/Lw × male AKR/Lw. These 2 groups were the most responsive to inoculation of PTA.

C3H/Bi strain.—Our C3Hf/Bi mice were obtained directly from Dr. J. J. Bittner in 1955, and have been inbred brother × sister in this laboratory for 9 additional generations. The incidence and types of neoplasms found in untreated mice from this strain were determined by examining a sample of 63 pedigreed mice representing the F81 to F83 generations, kept under observation until death. Fifteen neoplasms (24%) of reticular tissues appeared between the 11th and 18th months. Eight of these were lymphocytic neoplasms, 5 were type B reticulum-cell sarcomas, 1 a plasma-cell neoplasm, and 1 a type A reticulum-cell sarcoma, according to the classification of Dunn (*22*). In addition, the following neoplasms were found: 1 fibrosarcoma, 5 adrenal cortical adenomas, 1 adenoacanthoma of the mammary gland, 1 mesenchymal bone neoplasm, and 1 hemangioendothelioma.

(C3H × AKR)F_1 hybrids.—One hundred and forty-eight (C3H × AKR)F_1 mice were observed, throughout life, for neoplasms. Fifty-nine (40%) had reticular neoplasms, most of these of the lymphocytic type.

[5] Hereafter referred to as C3Hf/Bi mice.

The average age at death from this group of neoplasms was 15.0 months, the earliest one appearing at 6 months and the latest at 27 months. There were 33 neoplasms of mammary tissue among 79 females (42%), and 6 uterine and 2 ovarian neoplasms and 14 hepatomas among 69 males. In addition, 2 fibrosarcomas, 3 lung adenomas, 2 adrenal cortical adenomas, and 3 neoplasms of the kidney were observed. The potential for developing osteomas is great among females in this hybrid, especially in those beyond the age of 14 months. Incidences of 50 to 100 percent in different samples have been observed by Potter (*23*).

AKR/Lw strain.—The incidence of lymphocytic neoplasms, principally thymic in origin, for a sample of this substrain comprising inbred generations F42 to F49 was 92 percent, with death from the disease occurring at an average age of 8.0 months. The mean age at death from leukemia among males was nearly 1 month later than among the female group.

II. Tissue Sources

(*a*) *GLTC series.*—The leukemia used to prepare the $GLTC_1$ cultures was obtained from Dr. Ludwik Gross in December, 1956. It arose in a C3Hf/Gs mouse in Gross's experiment 3839 B. We received the leukemia in its original C3Hf/Gs host, and transplanted it to our C3Hf/Bi mice by intraperitoneal route. Explants for tissue culture were taken from the mesenteric lymph node of a mouse bearing the sixth transfer generation.

Cultures designated $GLTC_2$ were prepared from the same transplanted leukemia as $GLTC_1$, but explants were taken from the mesenteric node of a C3Hf/Bi mouse bearing the tenth transfer generation.

Similarly, cultures designated $GLTC_3$ were prepared from the eleventh transfer generation of this neoplasm in a C3Hf/Bi mouse.

This leukemia line was transferred serially in C3Hf/Bi mice up to the 20th generation when for unknown reasons it failed to grow progressively and to kill.

(*b*) *L-10813.*—This leukemia in a C3H/Gs host from Gross's "Passage A" series was received from Dr. Gross (*24*). Cultures were prepared with explants from the mesenteric node of the submitted host by the same method used in $GLTC_1$. This cell line was lost at the 31st transfer, again for unknown reasons.

(*c*) *L-12289.*—This leukemia in the original C3Hf/Gs host was received from Dr. Gross and represented his "Passage A" series #12, experiment 4194-d. Cultures were prepared with explants from the mesenteric node of the submitted animal, by the same method as with $GLTC_1$ and L-10813. At preparation of this manuscript, L-12289 has been transferred serially in C3Hf/Bi mice through 33 generations. Evidence will be presented later to indicate a loss of parotid-tumor-inducing activity from this serially transplanted lymphocytic neoplasm.

(*d*) *TT-427.*—Explants for these short-term cultures were obtained from a thymic epithelial tumor that arose in a C3Hf/Bi mouse that had received a filtrate of $GLTC_3$ cultures shortly after birth. The donor

also had bilateral parotid tumors at autopsy. The culture technique was the same as with $GLTC_1$.

(*e*) Additional neoplastic and non-neoplastic tissues tested by the short-term culture method are listed and identified in table 1. All were cultured by the same technique used for $GLTC_1$.

TABLE 1.—Tumors of parotid gland in mice receiving materials from short-term cultures of various neoplasms and normal tissues

Material	Donor strain of tissue	Number of surviving mice*	Number of litters	Number with parotid tumors	Latent period (*months*)
Normal spleen	StoLi	10	2	1	9.0
Normal spleen	C3Hf/Lw	8	2	5	2.5–12.0
Normal spleen	NH/Lw	9	3	None	—
Normal spleen	C3H/Bi	23	5	None	—
Normal spleen	AKR/Lw	32	5	None	—
Normal thymus	AKR/Lw	79	11	1	7.0
SL-1 lymphocytic leukemia	DBA/2	16	3	None	—
L1210 lymphocytic leukemia	DBA/2	46	12	1	9.0
Induced lymphocytic neoplasm	C3Hf/Bi	27	5	None	—
P815 mast-cell neoplasm	DBA/2	16	3	None	—
Bone tumor (GLBT)	C3Hf/Bi	21	5	None	—
P1081 granulocytic leukemia	DBA/2	14	3	None	—
Mammary tumor†	C3Hf/He	31	4	None	—
Mammary tumor‡	C3H/He	59	12	None	—
Total	.....	391	75	6	—

*C3Hf/Bi and (C3H × AKR)F_1 mice, principally, used as recipients. Total discard fluids and final cell scrapings after 2 to 4 weeks in culture inoculated without prior centrifugation or filtration. These results cover an observation period of 12 to 15 months.

†Mammary tumor from C3H subline freed of mammary-tumor agent.

‡Mammary tumor from C3H subline carrying mammary-tumor agent. Both neoplasms obtained from Dr. W. E. Heston, National Cancer Institute.

III. Tissue Cultures

(*a*) *Short-term.*—Cultures of the tissues and neoplasms listed in tables 1 and 2 were prepared with a modification of the Leighton sponge-matrix method previously described in the isolation of a reticulum-cell sarcoma (*25*). Cultures $GLTC_1$, $GLTC_2$, and $GLTC_3$ (table 2) were set up in roller tubes, and rotated at a rate of 12 revolutions per hour. All others were set up in flat-sided (Leighton) tubes.

The techniques used in $GLTC_2$ and $GLTC_3$ deviated from that used in $GLTC_1$ and the other cultures. In $GLTC_2$, no plasma clot or gelatin sponge was used, and the explants were simply allowed to drift in the medium as the tubes rotated. In $GLTC_3$, the gelatin sponge was omitted, but the explants were attached to the glass by plasma clot in standard roller-tube fashion.

All cultures were incubated at $35.5° \pm 0.5°$ C. The medium used for all cultures was LN40, whose components and preparation have been previously described (*25*). Feedings were generally performed 3 times weekly, with some variation according to rate of *p*H depression. The intervals at which

medium was saved and used for injection of animals are indicated in the tables. At each collection, 0.75 ml. of medium was removed from each tube and replaced with an equal amount of fresh medium, while 0.25 ml. of "conditioned" medium was left in the tube to keep the fluid volume at 1.0 ml. Six to 8 cultures were prepared in each explantation series.

(*b*) *Continuous culture line P388 D_1.*—The lymphoid neoplasm P388 has not been described in the literature, except with reference to its changes in tissue culture (*19*). A detailed description is in preparation. Briefly, P388 was induced by Dr. Michael Potter in a DBA/2 mouse, with skin paintings of methylcholanthrene. In the original host and in the early transfer generations, it was of moderately well-differentiated lymphocytic type. It was converted to ascitic form in the first mouse transfer and, during subsequent passages, underwent a rather extensive progression

TABLE 2.—Frequency of parotid tumors in mice receiving various types of tissue-culture material

Material	Number of mice	Number of mice surviving over 1 month	Number of mice showing parotid tumors	Number of litters (*positive/total*)	Type of preparation
$GLTC_1$	135	92	52	9/17	Filtered
$GLTC_2$	169	111	2	2/21	Filtered
$GLTC_3$	103	61	13	3/14	Filtered
L10813	168	49	1	1/15	Filtered
L12289	92	43	30	8/8	Not filtered
TT-427	130	65	8	3/13	Not filtered
P388	105	71	8	4/12	*See* table 10
P388 D_1	163	101	3	2/18	*See* table 11
PTA-1 in P388 D_1	314	166	109	21/30	Filtered
PTA-3 and -5 in P388 D_1	96	71	50	8/10	PTA-3 filtered PTA-5 not filtered
PTA in direct filtrate	82	21	0	0/4	Filtered
Medium alone (LN40)	73	64	2	1/11	Not filtered
Total.....	1630	915	278		

toward a poorly differentiated or stem-cell type of lymphoma. Mouse passage was entirely through (BALB/c × DBA/2)F_1 hybrids. At the 49th transfer, isolation of a continuous cell strain in tissue culture was established. The morphology of the continuous culture strain was definitely altered in comparison with the parental line, but the cultured cells retained the ability to develop into ascites tumors in mice, as well as the specific transplantability characteristics of the DBA/2 neoplasm.

In the present work, two types of cultures of P388 were studied. That designated P388 consisted of short-term cultures prepared by the same technique used for $GLTC_1$, with explants taken from tumor of the 60th mouse passage.

Cultures designated P388 D_1 represent cultures of the continuous, altered culture strain of P388. In these, the cells were grown on glass in the same medium (LN40) specified for all other cultures in this work.

IV. Derivation of PTA

Filtrates of cultures TT-427 were injected into newborn (C3H × AKR)F_1 mice to test for tumor-inducing activity. Eight of 47 animals eventually developed parotid-gland tumors, and the tumor from one of these (#904) was used to prepare a filtrate. This filtrate was then used to inoculate cultures of P388 D_1 at the start of serial passage of PTA through the continuous culture line. It is one of the strains also reported on by Rabson and Legallais (*20*). The derivation of PTA is represented diagrammatically as follows:

$GLTC_2$ (Leukemia) in C3Hf/BiLw	Tissue culture → Filtrate	Thymic tumor 427 in (C3Hf/Lw × AKR/Lw)F_1	Tissue culture → Filtrate
Parotid tumor 904 in C3Hf/BiLw	Direct → Filtrate	PTA	

Preparation of extracts and filtrates.—Discard fluids from the tissue-culture preparations were collected at the times indicated in the tables, and frozen immediately to −70° C. In some instances (also indicated in tables), several collections were pooled after thawing, just prior to inoculations, and were then refrozen for later use. In several experiments, dilutions with Locke's solution to 1:10 or 1:100 were made. These are mentioned specifically in the tables or text. Filtration, when done, was accomplished through a Selas #02 or #03 filter. All filters were tested with *Escherichia coli* during filtration.

Histologically diagnosed leukemic tissues, obtained from the sources mentioned previously, either from the original animal or from mice bearing intraperitoneal transplants, were removed aseptically. The tissues usually included spleen, liver, thymus, and superficial and internal lymph nodes. These were minced, then ground with mortar and pestle. A 20-percent cell suspension was prepared with chilled 0.85 percent sodium chloride or Locke's solution. Centrifugation was accomplished in a PR-2 refrigerated centrifuge for 10 minutes at 3000 rpm (1400 × g) and the supernatant fluid then recentrifuged at 9500 rpm (7000 × g) for 10 minutes. Filtration was accomplished through an *E. coli*-tested Selas #02 filter. This filtrate was then used immediately for inoculation into mice.

All inoculations were done subcutaneously, unless otherwise specified. A 26-gauge, 1½-inch needle was introduced through the upper muscles of the hind leg so that leakage was minimized; 0.05 ml. of the test material was deposited in the region of the nape of the neck. Mice were returned to their mothers and remained with them in separate cages until weaning. In some cases toxicity of the material was severe. This is noted in the text and tables.

V. Autopsies

The animals were killed when tumors became so large as to interfere with eating, when tumors were ulcerated with consequent bleeding and infection, or when dyspnea secondary to thymic tumors became severe. A small number of mice died unpredictably. These were autopsied when *post-mortem* changes were not extreme.

A detailed necropsy was performed on each animal, with the following organs taken for study: heart, lungs, liver, both kidneys, spleen, pancreas, thymus, both adrenals, thyroid, stomach, duodenum, ileum, submaxillary, sublingual, and parotid salivary glands on both sides, both extraorbital lacrimal glands, mammary tissue from axillary and inguinal regions, brain, pituitary gland, lymph nodes (cervical, mediastinal, axillary, brachial, inguinal, mesenteric, lumbar, and caudal), and spine and sternum for bone marrow study. In the male, testes, seminal vesicles, and preputial gland were taken, and in the female, uterus, tubes, ovaries, and vagina. In addition, skin and bone specimens were taken from locations bearing gross lesions. During the later part of the study, after the propagation of the agent in the continuous P388 D_1 cultures, complete coronal sections through the cranium and mandible were taken, as well as step-serial sections of the larynx and thyroid, at 300-micron intervals. Tissues were fixed in Zenker-formol, and stained with hematoxylin and eosin. Osseous tissues were decalcified in 5 percent formic acid.

RESULTS

Tables 1, 2, and 3 summarize the results in terms of positive or negative response with respect to parotid-tumor development. This criterion has been selected arbitrarily, though other types of tumors are found in many animals. In the present study, tumors of the special types related to the PTA were not found unless parotid tumors were present. Parotid tumors were sometimes present in the absence of other types. The latter therefore appear to represent the most sensitive indicator of activity of the agent, or agents, with which we are concerned.

It is important to note that the data presented in tables 1, 2, and 3 are not based on results in animals of a single strain, and direct comparisons are not justifiable without taking this into consideration. Details with regard to strains of animals used and the type of material injected are given in tables 4 through 12. Table 13 shows that all strains of mice used in this work can develop salivary-gland tumors of the type under consideration.

Although cultures $GLTC_1$, $GLTC_2$, $GLTC_3$, L-10813, and L-12289 were all prepared from leukemic tissues from Gross's "Passage A" series (*24*), the results among these sources are at variance. Differences among $GLTC_1$, $GLTC_2$, and $GLTC_3$ might be explained either by the difference in techniques that were used in preparing these cultures or by the fact

TABLE 3.—Frequency of tumors in mice receiving direct filtrates or extracts of leukemic tissues

Material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of leukemias	Number of parotid tumors	Number of other tumors	Mean latent period* (*months*)
(GLTC–1, 2, and 3) filtered	C3Hf/Bi	29	14	1 (coexisting with parotid tumor)	5	2 (fibrosarcomas)	Leukemia 4 Parotid tumor 6.5 Fibrosarcoma 9.0
L-10813 filtered	C3Hf/Bi (C3Hf × AKR)F_1	20	18	4 mice (in F_1)	0	0	Leukemia 5
L-12289 unfiltered	C3Hf/Bi	49	27	0	0	0	
L-12289 unfiltered	(C3Hf × AKR)F_1	19	16	0	0	0	
L-12289 unfiltered	C3Hf/Lw	33	30	0	0	0	
Total		150	105	5	5	2	

*Twelve-month follow-up period for entire group.

TABLE 4.—Frequency of tumors in mice receiving material from cultures $GLTC_1$

Age of culture (*material filtered*)	Strain of mouse	Number of mice	Number of mice surviving	Number of litters (*positive/total*)	Number of mice with parotid tumors	Latent period (*months*)	Tumor sites other than salivary and lacrimal gland group	
2 day	(C3Hf × AKR)F_1	42	35	3/5	23	3. 5–9. 0	Thymus	4
							Bone	2
							Mammary gland	2
8 day	(C3Hf × AKR)F_1	8	8	1/1	6	2. 5–3. 5	Thymus	1
							Mammary gland	1
							Subcutaneous connective tissue	1
2 day	C3Hf/Bi	14	4	1/1	3	3–5	Thymus	2
							Bone	2
							Mammary gland	3
							Subcutaneous connective tissue	1
							Hair follicles	1
4 day + 6 day	C3Hf/Bi	18	10	2/3	4	2. 5–5. 0	Mammary gland	1
							Bone	1
8 day	C3Hf/Bi	16	5	0/1	0	—	—	
13 day	C3Hf/Bi	19	14	1/3	6	2. 5–3. 0	Bone	2
							Mammary gland	5
							Subcutaneous connective tissue	2
							Hair follicles	1
							Adrenal medulla	1
							Renal medulla*	1
							Oral epithelium	2
13 day	C3Hf/Lw	18	16	2/3	10	5. 5–15. 0		
Total		135	92	10/17	52		Thymus	7
							Bone	7
							Mammary gland	12
							Subcutaneous connective tissue	4
							Hair follicles	2
							Adrenal medulla	1
							Renal medulla	1
							Oral epithelium	2

*Bilateral testicular lesions in one animal.

Adrenal medulla	1
Renal medulla	1
Oral epithelium	2

*Bilateral testicular lesions in one animal.

TABLE 5.—Frequency of tumors in mice receiving material from cultures $GLTC_2$

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor*	Number of litters (*positive/total*)	Latent *period*† (*months*)
1 day—discard medium (filtered)	C3H/Lw	32	23	0	0/4	—
	C3H/Bi	29	18	1‡	1/4	5
	(C3Hf × AKR)F_1	6	0	—	—	—
4 day—discard medium (filtered)	C3H/Lw	8	0	—	—	—
	C3H/Bi	8	7	0	0/2	—
	(C3Hf × AKR)F_1	7	7	0	0/1	—
6 day—discard medium (filtered)	C3H/Lw	11	8	0	0/1	—
	C3H/Bi	16	10	0	0/2	—
8 day—total culture (filtered)	C3H/Lw	18	13	0	0/2	—
	C3H/Bi	6	4	0	0/1	—
	(C3Hf × AKR)F_1	6	6	0	0/1	—
	DBA/2	9	9	0	0/1	—
8 day—total culture (centrifuged only)	C3H/Bi	5	5	1	1/1	14
	DBA/2	8	1	0	0/1	—
Total		169	111	2	2/21	

*Neither of the 2 mice bearing parotid tumors had tumors of any other organ.

†Total observation period of 15 months or more for all animals.

‡A portion of parotid tumor (H-44536) from this animal was removed aseptically and given to Dr. Wallace Rowe. It is the source of agent designated LID-1 by Dr. Rowe.

TABLE 6.—Frequency of tumors in mice receiving material from cultures $GLTC_2$

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal gland group	
1 day—discard medium (filtered)	(C3Hf × AKR)F_1	26	12*	9	2/2	3–14	Thymus	1
							Subcutaneous connective tissue.	2
1 day—discard medium (centrifuged only)	C3Hf/Bi	16	6†	0	0/3	—	—	
3 day—discard medium (filtered)	C3Hf/Bi	7	5	0	0/2	—	—	
3 day—discard medium (filtered)	C3Hf/Lw	26	22	0	0/4	—	—	
6 day—total culture (centrifuged only)	C3Hf/Bi	28	16‡	4	1/3	3–3. 5	Mammary gland	1
							Subcutaneous connective tissue.	1
Total		103	61	13	3/14	—	Thymus	1
							Mammary gland	1
							Subcutaneous connective tissue.	3

*Seven mice 48 to 72 hours old at injection; 4 developed parotid tumors.
†Three mice 48 to 72 hours old at injection.
‡Seven mice 48 to 72 hours old at injection; none developed parotid tumors.

TABLE 7.—Frequency of tumors in mice receiving material from cultures of L-10813

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)
3 day—discard medium (filtered)	C3H/Bi	10	3	0*	0/2
8, 12, 17, and 19 day—combined discard medium (filtered)	C3H/Bi	6	0	—	—
	SWR	7	1	0	0/1
	DBA/2	6	0	—	—
23 and 28 day—combined discard medium (filtered)	(C3Hf × AKR)F_1	10	0	—	—
	C3H/Bi	8	0	—	—
	DBA/2	7	3	0	0/2
	SWR	9	0	—	—
3, 5, 8, 12, 17, 19, 28, and 45 day—combined discard medium (filtered)	C3H/Bi	12	0	—	—
	(C3Hf × AKR)F_1	7	0	—	—
	SWR	5	2	0	0/1
18 day—total culture (filtered)	C3H/Bi	49	21	0	0/5
	C3H/Fg	17	7	1†	1/2
	(C3Hf × AKR)F_1	7	6	0	0/1
	SWR	8	6	0	0/1
Total		168	49	1	1/15

*Three generalized leukemias observed here. Filter found to be cracked.

†Latent period 5 months. Observation period for all mice was 12 months.

TABLE 8.—Frequency of tumors in mice receiving cultures of L-12289

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal gland group*	
2 day+5 day—discard medium (not centrifuged or filtered)	C3Hf/Bi	17	7	7	1/1	2. 5		
Same but frozen 20 days	C3Hf/Bi	11	8	4	2/2	3. 5		
	(C3Hf × AKR)F_1	9	9	4	1/1	3–4	Thymus	5
							Bone	18
							Mammary gland	6
27 day—discard medium (not centrifuged or filtered)	C3Hf/Bi	29	10	9	2/2	2	Subcutaneous connective tissue	9
							Hair follicles	3
							Adrenal gland	2
							Renal medulla	2
14 day—total culture (not centifuged or filtered)	C3Hf/Bi	10	0	—	—	—	Gingival epithelium	9
							Urethral glands	1
	AKR	7	3	2	1/1	2. 5		
	(C3Hf × AKR)F_1	9	6	4	1/1	2. 5		
Total		92	43	30†	8/8			

*Data based on only 22 animals in which histologic examination was complete.

†A parotid tumor from one of these mice was given to Dr. Wallace Rowe and is the source of agent designated by him as LID-2.

TABLE 9.—Frequency of tumors in mice receiving material from cultures of thymic tumor 427

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal glands
3, 6, and 11 day—discard media combined with total culture of 16th day (filtered)	{C3Hf/Bi + (C3Hf × AKR)F_1}	45	22	2*	1/5	2–3	None
Same, but after frozen at −70° C. 5 months	{C3Hf/Bi + (C3Hf × AKR)F_1}	85	43	6	2/8	2–2.5	None
Total		130	65	8	3/13		None

*A parotid tumor from one of these C3Hf/Bi mice (H-44583) was used for the preparation of PTA filtrate described under "Methods."

TABLE 10.—Frequency of tumors in mice receiving material from short-term cultures of P388

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period* (*months*)	Other tumors	
14 day—discard medium (filtered)	AKR	37	26	4	3/4	3–3.5	Mammary	1
17 day—discard medium (filtered)	DBA/2	16	10	0	0/2	—		
	C3Hf/Lw	11	11	0	0/2	—		
	StoLi	9	4	0	0/1	—		
21 day—discard medium (vibrated in paper jogger, frozen 9 months)	(C3Hf × AKR)F_1	26	14	0	0/2			
30 day—total culture	C3H/Bi	6	6	4	1/1	2.5–5.0	Mammary	2
							Fibrosarcomas	2
Total		105	71	8	4/12			

*Observation period for this group exceeds 9 months.

TABLE 11.—Frequency of tumors in mice receiving material from continuous cultures of P388 D_1

Age of culture and type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal glands
Medium LN40 alone	C3H/Bi AKR (C3Hf × AKR)F_1	73	64	2	1/11	5	Leukemia at 4th month.... 1
P388 D_1, 9 months—discard medium (filtered)	DBA/2	18	14	0	0/2	—	
P388 D_1, 9 months—discard medium (centrifuged only)	C3H/Bi	53	20	1	1/4	4	
P388 D_1, 8 months—discard medium (filtered)	C3H/Bi	6	0	—	—	—	
P388 D_1, 8 months—discard medium (filtered)	C3H/Lw	8	4	0	0/1	—	
P388 D_1, 9 months—discard medium (centrifuged only)	(C3Hf × AKR)F_1	9	9	0	0/1	—	
	SWR	6	0	—	—	—	
P388 D_1, 9 months—discard medium (filtered)	AKR	6	5	1	1/1	4	
P388 D_1, 9 months—1200 r—discard medium (centrifuged only)	C3H/Bi	4	4	0	0/1	—	
	(C3Hf × AKR)F_1	24	21	0	0/3	—	
	SWR	13	10	0	0/2	—	
Combined P388 D_1 and P388 D_1 1200 r—discard medium (centrifuged only)	C3H/Bi	3	2	1	1/1	—	Subcutaneous fibrosarcoma
	SWR	7	6	0	0/1	—	
	DBA/2	6	6	0	0/1	—	
Total*		163	101	3	3/18		

*Omitting controls LN40.

*Omitting controls LN40.

TABLE 12.—Frequency of tumors in mice receiving cultures of P388 D_1 exposed to parotid-tumor agent

Type of material	Strain of mouse	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal gland groups			
PTA (filtered extract of parotid tumor)	C3H/Bi (C3Hf × AKR)F_1	82	21	0	0/4	—	—			
P388 D_1 1 and 3 days after inoculation (discard medium not filtered)	C3H/Bi	17	16	8	2/3	2.5–8.0	Thyroid	2		
							Thymus	4		
							Bone	2		
							Hair follicle	2		
P388 D_1 6, 8, and 10 days after inoculation (discard medium not filtered)	C3H/Bi (C3Hf × AKR)F_1	25	18	16	2/3	1.5–2.5	Thymus	11	Hair follicle	5
							Thyroid	6	Renal medulla	1
							Mammary gland	2	Urethral gland	1
							Bone	5		
P388 D_1 14 days after inoculation (total culture—not filtered)	C3H/Bi (C3Hf × AKR)F_1	60	40	31	5/7	1.5–2.5	Thymus	28	Hair follicle	23
							Thyroid	17	Renal medulla	4
							Mammary gland	13	Oral epithelium	1
							Bone	19		

TABLE 13.—Strain susceptibility of various inbred strains of mice to PTA*

Strain	Number of mice	Number of mice surviving 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)	Tumor sites other than salivary and lacrimal glands	
(C3H × AKR)F_1	7	7	7	1/1	1. 8	Thymus	36
DBA/2Lw	16	11	11	2/2	2. 9	Bone	19
C57BR/cd	16	6	1	1/1	2. 5	Mammary	10
StoLi/Lw	14	4	3	1/1	3. 7	Thyroid	25
C3Hf/Fg	31	5	1	1/2	1. 5	Hair follicle	14
C3Hf/Gs	12	0	—	—	—	Renal medulla	2
C57BL/Ka†	18	12	1	1/2	2. 5	Adrenal medulla	1
AKR/Lw	21	10	10	2/2	1. 37	Nasal epithelial lesion	8
RFM	14	13	11	2/2	1. 9	Harderian gland	5
C58/Lw	5	5	4	1/1	2. 5	Bronchial epithelial lesion	2
SWR†	7	5	0	—	—	Subcutaneous connective tissue	1

*Culture fluids used from first passage of PTA on P388 D_1 leukemic cells, diluted 100 : 1.

†Resistance of C57BL/Ka strain to carcinogenic effects of PTA was again observed among a second group of 29 (4 litters) mice inoculated with the same material. Only 2 parotid-gland tumors in 6 months have appeared. In contrast, 5 of 10 (2 litters) SWR mice of a second group developed bilateral parotid-gland tumors at 2.8 months.

that, although the same leukemic line was used in each, the tissue came from different transfer generations (sixth, tenth, and eleventh, respectively). It would, however, seem unusual that tissue from the tenth transfer generation ($GLTC_2$) should have practically no activity, while tissue from the eleventh transfer generation ($GLTC_3$) had a moderate degree of activity. We are inclined to attribute the observed differences mainly to the technical differences used in the cultures. Thus $GLTC_2$, in which the explants were not attached either to glass or to gelatin sponge, gave the lowest tumor-inducing activity. Survival of the leukemic cells and the normal macrophages was also poorest in these cultures, as estimated by daily direct microscopic examination.

Known technical differences in the cultures do not explain the difference in results between cultures of L-10813 and L-12289. The latter produced tumors in 70 percent (30/43) of the animals, whereas the former produced tumors in only 2 percent (1/49) of the recipients. Two distinct differences between these 2 source materials are to be noted: (1) The tissues came from mice bearing different primary leukemias, though both leukemias were from the original C3H/Gs hosts that had received filtrate from Gross's leukemia agent in serial passage. (2) Probably of less importance, material from cultures of L-10813 was filtered, whereas that from L-12289 was not. Thus, filtration of the $GLTC_1$ cultures permitted a result differing only slightly from that obtained without filtering L-12289 (56% *vs.* 70%). Another difference in results between these same two sources when filtrates of extracts of the leukemic tissues themselves were used, without being cultured, is shown in table 3. Although no salivary-gland tumors were produced by either material, leukemias occurred in 4 of 18 C3Hf × AKR mice at less than 6 months of age after they had received filtrates of L-10813, while no leukemias were observed in 16 mice of the same hybrid constitution more than 12 months after they had received supernatant of L-12289. In this study, this is the only instance in which any suggestion of leukemia-inducing activity has been observed in an extract of leukemic tissue. The numbers of animals involved are too small to permit definite conclusions.

Parotid-gland and other tumors appeared in 8 of 71 mice receiving filtrates from short-term cultures of P388 (table 10). Although this is not a high proportion of positive results (11%), it is approximately 4 times higher than that obtained with the continuous P388 D_1 cultures, or with medium LN40 alone. Further work is needed with this tumor, as it is the only lymphoma of DBA/2 origin that has shown evidence of yielding salivary-gland tumor agent. It is also the only hydrocarbon-(methylcholanthrene) induced lymphoma that has shown such evidence. Unfortunately, we have no information at present as to the activity of direct filtrates of P388 tumor extracts. It is possibly relevant that the original DBA/2 host bearing the P388 lymphoma was born and reared in the Law colony, on the same floor but in a different room from animals carrying the Gross leukemias. On serial transfers, however, P388 was

passed through (BALB/c × DBA/2)F_1 animals reared in the central breeding laboratories supervised by Dr. Jay.

Comparison of the results in table 3 with those in table 2 for $GLTC_1$, L-10813, and L-12289 (in which the culture techniques were identical) provides only incomplete support for the thesis that culture of the source tissues by the method described makes the presence of the agent more readily detectable. In L-12289, there is a pronounced difference between the results with tissue-culture material and those with direct filtrate of the leukemic tissue. In the former, 70 percent of the 43 test animals bore parotid tumors, while none of 111 animals receiving direct filtrate showed tumors. In L-10813, however, no significant activity was demonstrable either with tissue-culture material or with direct filtrate of leukemic tissue. This result indicates, though it does not prove, that no agent was present in L-10813.

With $GLTC_1$, too few animals were tested with direct filtrate to permit a valid comparison with culture material. Although 5 of 14 animals receiving the direct filtrate bore salivary-gland tumors, all these positive animals belonged to a single litter. With such a small number of animals, the "litter effect" could obviously weight the results in a misleading manner. However, there appears to be value in the observation that among 92 animals, the proportion (56%), showing tumors after receiving cultures of $GLTC_1$ was about that (70%) found in 43 mice receiving cultures of L-12289. That the $GLTC_1$ culture material was filtered, while the L-12289 culture material was not, is a possible explanation for the minor difference in activity between these two sources.

The data in table 1 reveal that, in spite of a rather broad search for PTA in normal lymphoid organs and in a variety of transplanted neoplasms, scant evidence of its presence was found. The appearance of parotid tumors in 5 of 8 mice inoculated with cultures of normal spleen from a C3Hf/Lw mouse was to be expected in view of the observation that a high proportion of animals of this strain in the Law colony give positive reactions (*26*) for complement-fixing (CF) and hemagglutinatin-inhibiting (HI) antibodies against the PTA. Discard fluids from short-term cultures of 3 different 2-month-old AKR thymuses were used. HI antibodies were not detected in any of the donor AKR mice, nor were hemagglutinins observed in tissue cultures inoculated with extracts of the 3 transplantable DBA/2 leukemias used here: L1210, P815, and P1081. Thus, the occasional appearance of parotid-gland tumors may be expected, after introduction of materials, including tissue-culture medium alone (table 2), that do not themselves contain agent detectable by the available methods.

The negative results with mammary tumors of mice with or without the mammary-tumor agent provide reinforcement for our opinion, based on histologic differences, that the mammary tumors occurring in mice exposed to parotid-tumor agent differ from those associated with mammary-tumor agent as well as from those not associated with any known agent.

The most striking data in table 2 are those showing the results obtained with the continuous culture strain of P388 D_1 before and after exposure to filtered extract (PTA) of parotid tumor #904. Here it is shown that introduction of such filtrate into the cultures resulted in an increase of parotid-tumor incidence in recipient mice from 3 to 70 percent. A cytopathogenic effect was observed in the exposed cultures, as has been described by Rabson and Legallais (*20*), and illustrated in figures 33 to 37. Tumor-inducing activity in test animals was demonstrated through the tenth passage through P388 D_1 (table 14), and cytopathogenicity has likewise been observed through the fifteenth serial passage. In the serial passage of agent through P388 D_1 cultures, filtration with Selas #02 filter was performed on the inoculum at the third passage. In the remaining passages, frozen-thawed, unfiltered culture material was used.

It seems worth emphasis here that cells of the P388 D_1 strain were kept in the same growth medium used for their continuous propagation, both at the time of exposure to PTA and thereafter, until cytopathogenic effect (CPE) appeared and cultures were harvested. The 40 percent human-serum content of the medium did not prevent infection of the cells.

The first inoculation of a P388 D_1 culture was carried out by adding 0.15 ml. of Selas #02-filtered, 20 percent saline extract of parotid tumor #904 to a culture containing 5.0 ml. of medium. The culture was fed according to routine schedule by replacement of 4.0 ml. of medium on alternate days. After 18 days, no CPE had appeared, and the same culture was inoculated again with 1.0 ml. of the same filtrate previously used. After an additional 14 days and 5 feedings, a marked exfoliation of cells from the glass occurred, and this was harvested. The effective dilution of the second inoculum at this time was 1:18,750. In the 10 serial passages of the agent, it is calculated on the basis of dilution of the inoculums, initially and at feedings, that a total dilution of 1.5×10^{-39} was accomplished.

The times required for appearance of CPE in P388 D_1 cultures exposed to PTA in serial passages from the second to the tenth were 9, 8, 13, 7, 8, 9, 8, 9, and 9 days, respectively. From this it is apparent that, after an initial reduction in the latent period, no further effect occurred during this series of passages. The prolonged latent period for passage 4 is attributable to a 1:10 dilution of the inoculum.

Tumor-inducing activity appeared long before any CPE became apparent in the first passage of agent through P388 D_1. As shown in table 12, tumor-inducing activity was demonstrable in medium withdrawn from the first-passage culture within 3 days after exposure. Although activity increased with passage of more time, 100 percent tumor induction was never achieved. This would appear related to the previously described "litter effect," for tumors appeared in all or none of the members of each litter inoculated with culture material after CPE had appeared.

Table 14 presents the results of animal testing with the third, fifth, seventh, ninth, and tenth serial passages of PTA through P388 D_1 cultures. No significant differences are noted between the results in these passages

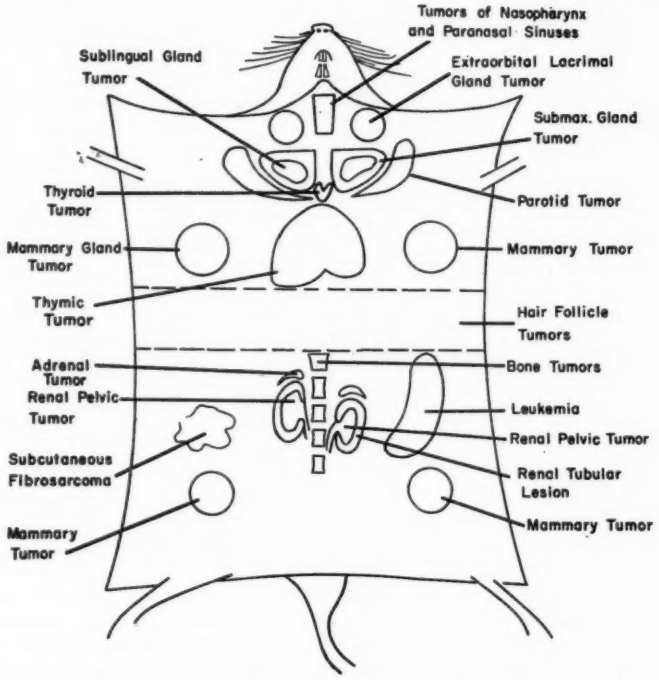
TABLE 14.—Frequency of tumors in mice receiving third to tenth serial passages of PTA in P388 D_1 *

Material	Mouse strain	Theoretical dilution of agent	Number of mice	Number of mice surviving over 1 month	Number of mice with parotid tumors	Number of litters (*positive/total*)	Latent period (*months*)
Third passage whole culture at CPE (filtered)	C3Hf/Bi	1.2×10^{-10}	37	23	16	3/4	1.5–2.5
Fifth passage whole culture at CPE	(C3H × AKR)F_1	7.9×10^{-16}	59	48	34	5/6	1.0–3.0
Seventh passage whole culture at CPE	(C3H × AKR)F_1	5.1×10^{-21}	49	9	4	1/1	1.0–4.0
	C3Hf/Bi			9	3	1/2	2.5
Ninth passage	C3Hf/Bi	3.6×10^{-27}	50	18	10	3/4	2.0–4.0
	(C3H × AKR)F_1			15	9	2/3	1.5–4.0
Tenth passage whole culture at CPE	(C3H × AKR)F_1	1.5×10^{-30}	47	29	15	4/5	1.5–3.5
Total.....	.....	.....	242	151	91	19/25	—

*Six of the 25 litters have remained tumor free. All 6 mothers had previously suckled litters receiving inoculations of virulent PTA and all litters presented tumors. Three of 6 mothers were HI positive at birth of these negative litters. Others were not tested. Mothers bearing positive litters had no previous litter or cage contact with PTA.

and those of the first passage. Histologic study of all animals receiving these later passages has not, however, been completed.

One of the outstanding differences between the observations on animals inoculated with P388 D_1 cultures exposed to PTA and those on animals inoculated with all short-term tissue cultures of leukemic material is that the latent period for appearance of tumors induced by the former was considerably shorter. Thus, before the agent was passed through P388 D_1 cultures, the shortest latent period ever observed was 2 months, and averaged more than 3 months. In all passages tested from 1 through 10,



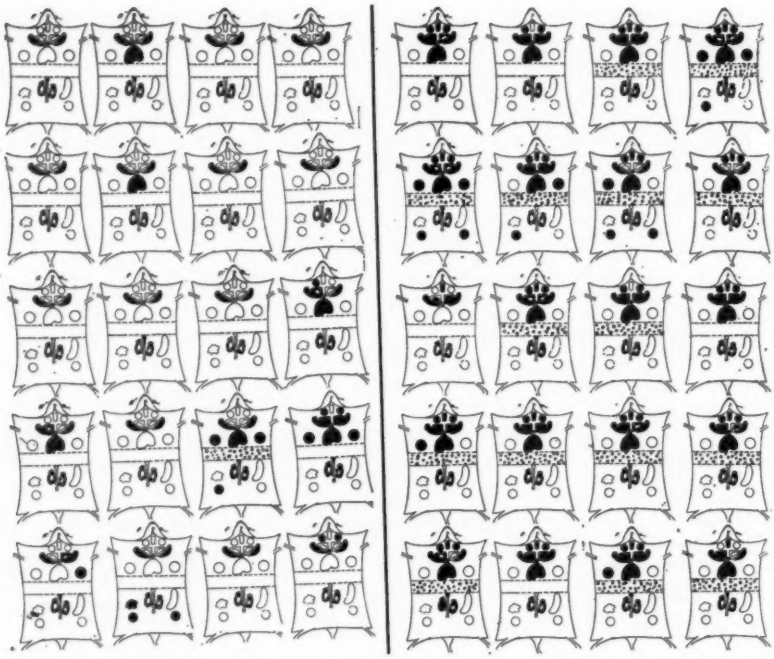
TEXT-FIGURE 1.—Key diagram, identifying organs and tissues involved by tumors or lesions in text-figure 2. Four foci for mammary tumors are arbitrarily used to depict multiple tumors of this tissue when observed.

the latent period was reduced to as little as 1 month, and averaged less than 2 months.

Another outstanding difference between animals receiving PTA from P388 D_1 cultures and those receiving all previous types of material is that the variety and number of tumors in the former was greater, as shown in table 15. In order to depict this difference diagrammatically, 20 consecutively autopsied C3Hf × AKR mice from the $GLTC_1$ experiment are compared in text-figures 1 and 2 with 20 consecutively autopsied C3H × AKR mice that had received the first passage of PTA in P388 D_1.

The variations in primary tumor sites from one individual animal to another, as well as from group to group, are self-evident in this diagram.

Although PTA has been shown by other investigators to be relatively stable in the frozen state, there are indications that loss or qualitative change in activity may occur during prolonged storage and repeated thawing and refreezing.



TEXT-FIGURE 2.—(*A*) *Left* of center line: Sites involved by tumors or lesions in 20 consecutive (C3Hf × AKR)F_1 mice that developed tumors of the parotid glands after inoculation with short-term tissue cultures of mouse leukemia ($GLTC_1$ series). Organs with tumors are represented in *solid black*, except for renal cortex, where *solid black* indicates severe renal tubular lesions and *stippling* indicates moderately severe renal tubular lesions. *Stippling* of the transverse strip indicates presence of hair-follicle tumors.

(*B*) *Right* of center line: Sites involved by tumors or lesions in 20 consecutive (C3Hf × AKR)F_1 mice that developed tumors of the parotid glands after inoculation with first passage of PTA through P388 D_1 culture. All tumors and lesions in groups A and B identified histologically.

Our data are not complete at present, but over a 9-month period of storage at −70° C., during which 15 samples were removed from one repeatedly thawed lot, there has been a definite decrease in the proportion of thyroid and hair-follicle tumors that appeared in association with parotid tumors. At the same time, there has been no decrease in the frequency or latent period of parotid tumors produced by the same preparation. This

TABLE 15.—Sites of primary tumors identified histologically in mice receiving (a) short-term cultures of $GLTC_1$, and (b) first passage of PTA in P388 D_1

Material	Animals inoculated and surviving over 1 month (*number*)	Organ or tissue site of primary tumor											
		Parotid gland	Extra-orbital lacrimal gland	Sub-maxillary gland	Sub-lingual gland	Thymus	Hair follicles	Thyroid gland	Bone	Mammary gland	Renal medulla	Subcutaneous connective tissue
$GLTC_1$	92	52	15	8	3	7	2	0	6	11	1	4
First-passage PTA in P388 D_1	112*	66†	60	55	44	44	31	27	26	15	5	1

*Includes only the mice inoculated with culture material after appearance of CPE. Strains were as listed in tables 12 and 13.
†Four additional mice had gross manifestations of parotid tumors but were not examined histologically.

observation simply reasserts the importance of storing this agent, like others, in individual ampoules for any quantitative experiment.

Strain Sensitivity

When it became clear that the harvested material of the first passage of PTA in P388 D_1 had high tumor-inducing potency, a sample of this material was diluted 1:100 and was used to inoculate a variety of strains of inbred mice to determine whether some strains may be refractory to the effects of the agent. In table 13, 10 inbred strains are represented, not including C3H/Bi and C3H/Lw, both of which had previously responded, though C3H/Lw has consistently shown a relatively prolonged latent period. It is evident from table 13 that every strain tested has shown positive evidence of susceptibility. Mirand *et al.* (*27*) have previously reported susceptibility of 4 strains and 1 F_1 hybrid to SE polyoma virus. It seems probable that relative differences in susceptibility exist among the strains, for until the agent passed through P388 D_1 was used, we had observed no response to other positive materials by DBA/2 or StoLi mice. It should be noted here, also, that Gross reported C57BR mice resistant to PTA (*28*). The appearance of characteristic parotid tumors in 1 of 6 C57BR mice in our tests indicates that animals of this strain are capable of response, though some degree of resistance may exist in C57BR as well as in C57BL/Ka. The question of relative strain susceptibility deserves further, more critical investigation, with the use of uniform, standardized preparations of agent.

Age Sensitivity

It is clear from the results shown in table 16 that sensitivity to PTA is demonstrable, at least in certain litters, through the first 14 days *post partum*, without appreciable lengthening of the latent period. Parotid tumors also appeared in 4 of 10 C3Hf/Bi mice that received intravenous inoculations, after weaning, between 30 and 40 days of age. The latent period in these 2 instances was 8 to 9 months. In connection with another study, we have recently observed parotid, thymic, thyroid, and hair-follicle tumors in mice exposed to PTA at 1 month of age and after re-

TABLE 16.—Age susceptibility of C3Hf/Bi and (C3H × AKR)F_1 mice to PTA*

Age inoculated	Number of mice surviving over 1 month	Number with parotid-gland tumors	Number of litters (*positive/total*)	Average latent period (*months*)
Under 24 hours	34	26 (80%)	5/6	1. 85
Under 48 hours	20	15 (75%)	2/3	1. 6
Four to 5 days	11	10 (91%)	2/2	1. 75
Thirteen to 14 days	14	7 (50%)	2/2	1. 75
Seventeen to 21 days	32	2	2/5	3. 0
Twenty-nine days	7	0	—	—

*Discard culture fluids from first passage of PTA on P388 D_1 leukemic cells used. Inoculum diluted 100 : 1. Material kept at −70° C. for 6 months prior to use.

ceiving 300 r of total-body X irradiation. The latent period for these tumors was less than 3 months. These results are in contrast to the findings of Gross (*29*) who suggested that age differences in response could be used to differentiate between the leukemia-inducing agent and the agent(s) responsible for the appearance of parotid-gland tumors. A more complete histologic study of the tissue response of 14-day-or-older mice, in contrast to younger mice, may reveal differences which are not now apparent.

Infectivity of Other Tissue-Culture Lines

We have as yet made only a few exploratory trials of other continuous tissue-culture cell strains in an attempt to ascertain their susceptibility to the CPE-producing effect of PTA. Both the P329 (*25*) and MB-III strains [6] showed CPE 5 and 10 days, respectively, after initial exposure to PTA of the fifth serial passage through P388 D_1. Cultures of both cell strains were harvested after appearance of CPE and tested for agent, with results shown in table 17. The tumor-inducing effectiveness of the agent after a single passage through these cell lines did not appear appreciably different from that observed with the P388 D_1 line.

All the continuous tissue-culture cell lines that have been found susceptible to infection with PTA have been derived from lymphomas, but this fact has little significance until many other cell lines are tested.

We have also inoculated cultures of normal macrophages isolated from the peritoneum of (BALB/c × DBA)F_1 mice after stimulation with sterile mineral oil. The cultures were inoculated 11 days after isolation, and did not show a clear-cut CPE. However, within 7 days after exposure, all the test cultures showed a definite decline in metabolic activity, as indicated by the rate of *p*H change in the test cultures, compared with

TABLE 17.—Tumors of parotid gland in mice receiving materials from control and PTA-infected continuous cultures

Material	Donor strain of culture cell line	Theoretical dilution of original inoculum	Number of surviving mice	Number of litters	Number with parotid tumor	Latent period (*months*)
Lymphosarcoma MB-III	(C57BL × DBA)		56*	11	2	4.5–5.0
MB-III with PTA	(C57BL × DBA)	1:1024	21	4†	12	2.0–4.0
Histiocytic neoplasm P329	DBA/2		36	6	1	8.0
P329 with PTA	DBA/2	1:32	20	3‡	8	1.0–2.5

*Entire fluids and cell culture used without centrifugation or filtration. Recipient mice were C3Hf/Bi and (C3H × AKR)F_1.

†Twelve mice from 3 litters presented tumors. All mice of the fourth litter were negative. The C3Hf/Lw mother had HI antibody > 200 at birth of litter and had previously suckled a litter receiving highly active PTA.

‡One litter of 8 mice had parotid-gland tumors. Mothers of 2 negative litters had HI titers > 200 at birth of litters.

[6] Originally isolated by Dr. W. M. DeBruyn from lymphosarcoma MB (T86157) in 1949 (*30*). Cultures of this strain were kindly provided us by Dr. G. O. Gey, and had been maintained continuously for 12 months in our laboratory in medium LN40 at the time of exposure to PTA.

that of uninoculated control cultures. Furthermore, the cultures exposed to the agent declined in population more rapidly and were completely lost more than a month earlier than the control cultures.

"Carrier" Cultures

After the appearance of CPE in the second serial passage of PTA in strain P388 D_1, one of the exposed cultures was not harvested, but was carefully treated by giving small, frequent feedings and by closely controlling the *p*H. It was possible to obtain a "carrier" cell line in this way, and this line was maintained for 9 months after the single exposure. It was subcultured several times without difficulty, though it chronically displayed some of the features characteristic of the early stages of CPE. The cells tended to aggregate in small groups that could be seen grossly and had the appearance of small curds on the walls of the tubes. Microscopically, these cell aggregates often had one or more large, apparently degenerating, giant cells within them. Also, smaller numbers of cells, usually about 8 to 12, formed rosettes on the glass, with 1 cell placed centrally and the remainder arranged in a cluster around it (fig. 35).

The effective dilution of PTA in this cell line as a result of feedings and subcultures during the 9-month period was approximately 1.0×10^{-42}. Seventy-two days after this "PTA carrier" cell strain had been exposed to PTA, at which time regrowth had been well established, one culture was frozen and thawed and, without filtering, injected in the usual manner into 11 (C3H $\times$ AKR)F_1 newborn mice and 6 C3Hf/Bi mice, comprising 3 litters in all. After 4 months of observation, all 17 animals showed gross evidence of salivary-gland tumors and tumors of the thymus. Six months after the initial infection, carrier cultures were again inoculated into newborn mice. Three of 26, in 2 of 4 litters, have shown tumors during the first 3 months of observation. It is apparent that the carrier cell line continued to yield the agent in considerable quantity, even though the cells were not killed at a rate sufficient to prevent their continuous propagation. It seems possible that the establishment of this carrier state of P388 D_1 cells *in vitro* was largely dependent on the high serum content of the medium. Rabson (*31*), using a nonserum-containing medium, has not been able to obtain recovery of P388 D_1 cultures once CPE has been produced by this agent. These observations are in keeping with known facts in other virus-carrying culture systems as recently discussed by Takemoto and Habel (*32*).

"Litter Effect"

Some data are available at present and a more detailed analysis will be given later of the "litter effect" described earlier (*1*) and noted also in this study. By reference to the last 2 columns of table 12 and to table 14 it may be seen that certain entire litters remained tumor-free after administration in the early neonatal period of the highly effective P388 D_1-propagated line of PTA. On the other hand, if one member of an in-

oculated litter developed a parotid-gland neoplasm, most or all litter mates did. It is quite clear, in retrospect, that the majority of mothers that had a previous experience of nursing newborn mice inoculated with the highly active P388 D_1-propagated line of PTA delivered subsequent litters completely resistant to infection by PTA. Of 17 C3Hf/Lw mothers (mated to AKR males) that had nursed previously inoculated litters developing tumors, 13 delivered offspring which showed the "litter effect"; that is, the inoculated mice uniformly failed to develop tumors. From 6 of these 17 C3Hf/Lw mothers serums were obtained for CF and HI antibody determinations. All contained high levels of antibody. Four females that nursed the inoculated newborn, among which only 1 or 2 per litter presented tumors, had second subsequent litters that were susceptible to PTA.

By reference to table 14, it may be seen that 6 entire litters of 25 have remained resistant, over a 6- to 9-month observation period, to the highly active PTA preparation propagated serially on P388 D_1 cultures. Each of the 6 mothers that bore a resistant litter had a previous history of exposure to PTA, through an infected (inoculated) suckling litter, and in 3 of the 6, HI antibodies were detected in the mothers at delivery of the litters that remained resistant through this observation period. Thus, in this series all litters and nearly 100 percent of the inoculated mice from uninfected mothers developed parotid tumors.

These results are in accord with the findings of Rowe *et al.* (*26*) that mothers nursing inoculated newborns were uniformly infected within 4 weeks of contact and that inoculated newborn mice are a source of intense environmental contamination.

It is apparent also that a certain number of mothers, which had had no previous contamination with inoculated litters, nor cage contamination with litter mates, also delivered litters which resisted infection with PTA. These probably represent "spontaneous convertors." A limited serologic experience with these has revealed that in each case showing positive serology (that is, HI $>$ 200), the litter has been consistently negative.

Observations on Tumor Incidence in Combined Litters

The observations of Rowe *et al.* (*26*) concerning spontaneous conversions of many strains and ages of mice to HI antibody producers indicate the importance of cage contact in the spread of parotid-tumor virus. In 10 instances in the present study 2 or more litters were caged together at weaning; in all instances, 1 litter in each cage had received highly virulent tumor-inducing material, usually from the P388 D_1-passaged line, while 1 or more litters received control inoculations or were inoculated litters from mothers serologically positive for HI antibodies that presumably protected the litter through the transfer of maternal antibodies. Although it is expected that most or all cage mates converted as a result of contact with those receiving the highly active materials, in not a single instance, over a 12-month observation period, was there observed induc-

tion of neoplasms among these control mice. Among the 10 cages there were 10 litters representing 42 mice inoculated within the first 48 hours of the neonatal period with virulent, tumor-inducing material. Forty of 42 showed parotid-gland neoplasms and associated tumors and lesions at 2.4 months. In contrast, their cage mates, comprising 37 mice of 12 litters that had received inoculums of control materials, failed to develop neoplasms.

It was possible to obtain offspring from both C3Hf/Bi and C3Hf/Lw mothers (the latter mated with AKR to obtain F_1 mice) that presented parotid-gland neoplasms while suckling their young. Eight litters of 46 offspring from 8 different C3Hf/Bi mothers, and 5 litters of 29 (C3Hf/Lw × AKR)F_1 mice from 5 separate C3Hf/Lw mothers were observed for 12 months. Neither parotid-gland neoplasms nor leukemia was observed in these mice. Of several of these tumor-bearing mice examined for HI antibodies, all gave extremely high antibody titers (> 5120). Similarly, mothers of these 2 strains, which were negative for neoplasms but whose litter mates presented parotid-gland neoplasms, produced offspring that were observed for 12 months and did not present neoplasms. Five litters, comprising 22 mice from C3Hf/Bi mothers and 20 (C3Hf/Lw × AKR)F_1 mice from 3 C3H/Lw mothers were observed for this period. This latter group of mothers was inoculated at birth with PTA.

It is of interest to point out also that as a result of a survey of the antibody status of various strains of mice in this laboratory. (*26*) there have been available for observation mice of the inbred strains DBA/2, C3Hf/Fg, AKR, C3Hf/Bi, and others having high HI antibody titers as a result of spontaneous infection by PTA, or as a result of cage infection by inoculated litter mates. Serologically, the former were found to convert spontaneously beyond the age of 3 months. To date, after an observation period of more than 8 months, at which time most of these mice have now reached the age of 11 to 16 months, no unusual morphologic forms or changes in incidence of the normally occurring "spontaneous" neoplasms have been seen.

Frequency of Leukemia in Recipient Mice

Rudali *et al.* (*33*) have observed what they call "superinfection" in high-leukemic AK mice given AK leukemic extracts (not filtrates) parenterally in the neonatal period, but apparently have not observed induction of lymphocytic neoplasms in their C3Hf mice. The outstanding feature of "superinfection" was the shortened period of latency, some leukemias appearing as early as 80 days. Maximum incidence was reached in the inoculated mice at 4.5 months and at 9.0 months in untreated controls. The claim of an increased incidence over controls is questionable. These extracts also produce other neoplasms, but particularly parotid-gland tumors, in AK and C3Hf mice, at times in conjunction with lymphocytic neoplasms in AK mice. Dulaney *et al.* (*15*) have observed a similar acceleration of leukemia in AKR mice.

We have not observed an earlier appearance of lymphocytic neoplasms in AKR/Lw nor in (C3H × AKR)F_1 mice in our present work. Among those AKR mice that received positive extracts, *i.e.*, the mice from litters in which at least 1 member presented parotid-gland and associated tumors, 17 of 27 AKR strain mice, comprising 6 litters, had lymphocytic neoplasms. The average age at death among females was 8.9 months (7–12 months), and among males 9.2 months (7–11 months). Since the other mice of this series either had parotid-gland tumors (and associated tumors) or were killed at 12 months of age, the precise incidence cannot be determined, but the average age at death from leukemia is in the same range as uninoculated AKR controls. Similarly, among 41 (C3H × AKR)F_1 mice, comprising 7 litters, of which at least 1 member of a litter presented parotid-gland neoplasms, there were 8 lymphocytic neoplasms appearing between 6 and 12 months at which time these (C3H × AKR)F_1 mice were killed. The mean age at death was 9.3 months. The distribution of leukemic deaths was similar to uninoculated controls. (*See* "Materials and Methods.")

The lack of correlation of PTA antibody (HI and CF) with the occurrence of lymphocytic neoplasms in AKR mice (*26*) also suggests that there is no etiologic relationship of PTA to spontaneous leukemias, at least for those appearing in the AKR strain. Further evidence of such a lack of correlation has been found also in X-ray-induced lymphocytic neoplasms of the C3Hf/Bi substrain of mice. Details of these studies will be published later (*34*).

Loss of Tumor-Inducing Activity From the Transplantable Lymphocytic Neoplasm L-12289

Discard media, obtained over a 2-week period during the short-term culture of the lymphocytic neoplasm designated L-12289, exhibited a striking tumor-inducing activity upon inoculation into appropriate sensitive recipients (70% parotid-gland neoplasms in 43 mice). (*See* table 8.) High hemagglutinin titers ($> 1:2560$) were obtained from these short-term culture media, and CPE was observed at 5 days when this material was introduced into mouse-embryo tissue cultures.[7]

After 20 successive serial passages of the neoplastic cells (leukemia) in 1- to 2-month old C3Hf/Bi mice, it was found that the neoplastic cells no longer gave evidence of carrying virus. Hemagglutination-inhibiting antibodies were not detected in terminally ill mice bearing the transplanted leukemia, neither CPE nor hemagglutinins were detected in mouse-embryo tissue cultures, and virus was not recovered after introduction into Swiss mice.[7]

In view of these findings, which are in contrast to the observations of Stewart *et al.* (*35*) and Buffett *et al.* (*12*) of persistent virus in transplantable AKR neoplasms, short-term cultures of L-12289 were again carried

[7] Personal communication from Dr. Isadore Brodsky, Institute of Allergy and Infectious Diseases, National Institutes of Health.

out with leukemic tissues of C3Hf/Bi mice bearing the 30th transfer of this leukemia. Twenty-seven C3Hf/Bi and (C3H × AKR)F_1 recipients, which received discard fluids from L-12289 in short-term culture, have remained tumor-free over a 6-month observation period.

Pathology

The gross and microscopic morphology of tumors of the salivary glands and extraorbital lacrimal gland has been described in detail in the earlier report of Law, Dunn, and Boyle (*1*). The tumors of these organs seen in the present work were not significantly different from those of the earlier experience, and will not be further described at this time.

Several types of tumors were observed in the present work, which had not been encountered at all in the previous study (*1*). These are described later. Table 18 lists the types and number of tumors or proliferative lesions examined. Tumors of the submaxillary, sublingual, and accessory salivary glands of the oropharynx, and tumors of the submucosal glands of the nasal passages and trachea are not listed or described, as they are similar to those of the parotid gland. Among the tumors, they were noted with a frequency second only to tumors of the parotid gland, though they were less frequent than the renal-cortical lesions.

It may be seen in table 18 that the organs involved by tumor were, with few exceptions, the same as those listed in the report of Stewart, Eddy, and Borgese (*36*). The various tumors listed and illustrated by these authors appear to be identical with those described later, though there are some differences in terminology. From this it appears that

TABLE 18—Tumors and proliferating lesions identified histologically in 232 mice developing salivary-gland tumors

Anatomic site	Number histologically positive for tumor or proliferative lesion*	
Parotid	232	
Thymus	93	
Bone	71	
Thyroid	51	
Hair follicles	50	
Mammary gland	45	(33 females) (12 males)
Subcutaneous connective tissue	22	
Oral epithelium	12	
Renal medulla	10	
Nasal mucosal surface epithelium	8	
Harderian glands	5	
Adrenal medulla	4	
Urethral glands	2	
Bronchial epithelium	2	

*Lesions of renal cortex, although often showing evidence of proliferative activity, not included. Such lesions were second only to parotid tumors in frequency.

propagation of the agent in a continuous culture cell strain derived from a lymphoma did not immediately result in obvious differences in its tumor-producing properties as compared with a similar agent propagated in normal mouse-embryo cultures. However, the long-term effect of propagating the virus on different cellular substrates cannot be predicted.

Thymic Tumors

These ranged in size from microscopic to 2.5 cm. in greatest dimension. The majority were recognized grossly, but several were discovered only at histologic examination. The largest ones occupied approximately three fourths of the intrathoracic space, providing ready explanation for the severe dyspnea associated with them during life. Even in these, it was usually possible to distinguish an anterior midline cleft demarcating the diffusely enlarged right and left lobes. In most instances the right lobe was larger than the left. There were often grossly visible necrosis and hemorrhage in the portions of tumor adjacent to the diaphragm. The surface was usually smooth and not adherent to the pleura. A cephalad extension through the thoracic inlet was occasionally present. The tumors were pearly white in color, with a prominent, dilated vascular system over the surface. Their consistency was soft, much like that of a lymphoid tumor.

Microscopically, a considerable degree of variation was observed in these tumors from case to case, as well as within a single tumor, but in general they were less pleomorphic than the salivary-gland tumors. Like the latter, they are considered to be of a single nosologic type. The proliferating cells varied from large, pale, round cells, through spindle forms, to cuboidal or slightly columnar cells with definite epithelial characteristics (figs. 4 through 8). Cells of the latter type appeared to represent a modulation of the other two types, and were found chiefly in palisaded arrangement around blood vessels, or in follicle-like formations around lumens filled with an eosinophilic, colloid-like material. In such follicle-like structures, clear spaces were sometimes present between the lining cells and the colloid-like material, as in follicles of the thyroid gland. A blood vessel was almost invariably identifiable within the follicle-like structure, usually eccentrically placed, and giving the impression that it had been separated from contact with the epithelial cells by the accumulation of the colloid material.

The periodic acid-Schiff reaction preceded by diastase digestion did not reveal any positively reacting material within the proliferating cells, either of the epithelial or the round and spindle-cell types. The extracellular colloid material reacted with a deep-pink color, more intense than that of plasma. The reticulin network, as revealed by PAS reaction and by Wilder's silver stain, showed no characteristic pattern, and the quantity of reticulin within the tumors was scant. Mallory's phosphotungstic acid method and van Gieson's connective-tissue stain did not show any fibrils within the spindled types of tumor cells, or of collagen deposition by tumor cells.

Thymic tumors displaying obvious epithelial characteristics, as described, were actually exceptions to the rule, but in the same sections there were many areas that showed the round and spindle-celled forms that exclusively made up most tumors. Intermediate types were also seen, which provided support for the impression that all the tumors were derived from thymic epithelium. The loss of epithelial character in most cells is in keeping with experience with thymic epithelial tumors in man (*37*). One of the constantly disturbing anatomic features of the thymus has been the difficulty in distinguishing thymic epithelium (the "epithelial reticulum") from reticulum cells of mesenchymal origin. Recently, Buffett *et al.* (*12*) have expressed the view that thymic tumors occurring in their experiments with PTA are of reticulum-cell origin. The tumors described by them appear to be histologically the same as those observed by us. We are of the opinion that the tumors in our experiments, and probably also those reported by Buffett *et al.*, originate from the thymic epithelium.

In most of the thymic tumors, the lymphocytic elements were usually nearly completely replaced by tumor. Extensions of the proliferating cells through the capsule, to form layers or nodules on the external surface, were common. Areas of necrosis, sometimes with calcification, were frequent.

In a small proportion of the tumors, lymphocytes dominated the picture numerically, though it was always possible to find the abnormal thymic epithelial proliferations amid the lymphoid masses. Morphologically, there was nothing about the lymphocytes or their distribution to suggest that they represented a leukemic or lymphomatous process. Rather, they appeared to represent a very marked reactive hyperplasia.

In some instances that appeared to represent the early stages of development of the thymic tumors, the lesions consisted of discrete nodules of proliferating cells, occupying either the medulla or cortex of the organ (fig. 2). In other instances, proliferation of altered epithelium appeared to be taking place simultaneously in many parts of the organ, particularly along the courses of the cortical blood vessels and beneath the capsule. This evidently represented diffuse, multifocal origin from the epithelial reticulum. The earliest example we have observed as yet was in a 21-day-old mouse, inoculated on the day of birth. As shown in figures 1 and 3, the diffuse epithelial proliferation in this instance is rather sharply limited to the cortex on the ventral side of the medulla. The medulla and dorsal cortex remain essentially unchanged. It is our impression that the thymic tumors originate from the less well-differentiated thymic epithelium around the vessels, rather than from the more differentiated thymic epithelium of the Hassall's corpuscles.

Finally, we have seen several thymic epithelial tumors taking origin from ectopic thymic tissue. Two of these were found high in the neck adjacent to salivary-gland tumors, and four just below and in contact with the lower pole of the thyroid gland. One of these (fig. 14) was

difficult to distinguish grossly from a primary thyroid tumor because it displaced and compressed one lobe of the thyroid gland.

Thyroid Tumors

As in other involved organs, lesions of the thyroid gland were usually multiple, involving both lobes, though occasionally solitary. The largest nodules measured 0.5 to 0.7 cm. in greatest dimension, whereas the smallest were microscopic and might be limited to a single follicle. Grossly, the smaller lesions could only be suspected on the basis of a certain degree of pallor and granularity of the gland. Under a dissecting microscope, these lesions were recognized as small, whitish nodules, slightly elevated above the pink thyroid parenchyma. The larger lesions were opaque and white, with smooth surfaces.

Microscopically, the earliest recognizable lesions consisted of one or several follicles within which some of the epithelial cells were altered, and around which were collections of lymphocytes and plasma cells (figs. 16 and 17). The follicular epithelial alteration was marked by enlargement of the cells, particularly the nuclei, with a relative loss of cytoplasm. Nuclei were enlarged to two or three times their usual size, and some nuclei contained large, clear, sharply circumscribed vacuoles that failed to take any stain (hematoxylin and eosin). Some cell necrosis was indicated by the nuclear debris within the involved follicles, and occasionally solid, eosinophilic areas of tumor necrosis were seen. In some follicles only a portion of the epithelium showed the alterations described, while the remainder appeared normal. Involved follicles generally contained less colloid than adjacent normal follicles. The lesions appeared to progress by proliferation of the large, altered cells, which formed papillary infoldings or accumulated in solid, expanding masses. The larger tumors contained, at most, only scant amounts of colloid in irregular spaces between tortuous folds of epithelium (fig. 15). Some of the larger tumors were completely solid, without signs of function or differentiation. Occasional examples of this type also contained rather large collections of lymphocytes in the midst of the proliferating altered epithelium.

The peripheries of the larger lesions deserving designation as tumors were sharply circumscribed, but not encapsulated. Extension beyond the capsule of the gland or invasion of blood vessels was not noted. Parathyroid tissue adjacent to tumor was pushed ahead of the expanding lesion, but was not invaded.

Mammary Tumors

In a previous publication (*1*) two subcutaneous epithelial tumors in a male mouse were described. The resemblance to the parotid-gland tumors was noted, and it was suggested that these tumors probably originated from "subcutaneous glandular elements." In the present experiment, 45 subcutaneous epithelial tumors, 33 in female and 12 in male mice,

have been found. Some of these had a close resemblance to the tumors previously described and the opportunity to examine them, often in early stages of development, left no doubt that the tumors were of mammary-gland origin. They differed, however, both in morphology and pathogenesis from the mammary tumors usually encountered in mice. Although a number of morphologic variants have been described, mammary tumors in mice usually reproduce mammary acini in some areas, and these acini were lacking in the tumors of the present series. Mammary tumors are seldom found, even in inbred mice with the milk agent, before the age of 6 months, and they have not previously been reported in male mice unless the males were treated with female sex hormones (*38*), or had developed a hormonal imbalance (*39*). Mammary-gland tissue is rudimentary in the male unless there is estrogenic stimulation. Yet mammary tumors in the present series were found in mice only 2 months old and a high proportion (29%) were in males. We have, therefore, in these treated mice a group of mammary tumors that differ in morphology and biologic behavior from mammary tumors previously studied. Mice in the present experiment with mammary tumors were all from inbred strains or hybrids, the milk agent was absent, and the tumors appeared at a much earlier age than the usual milk-agent tumors. Mice receiving injections at birth, as shown in the previous experiment, may develop mammary tumors of the usual types, provided they live to the required age. This may explain why, in some reports on mice receiving the PTA when newborn, the mammary tumors described have not been recognized as different from the usual types.

The mammary tumors in the present group of mice appeared to originate in small epithelium-lined cysts or tubules (fig. 9). These structures were often lined by two or more layers of epithelial cells. The inner layer next to the lumen was composed of basophilic cuboidal cells, while the cells of the outer layer were paler and more oval. The lumens often contained an eosinophilic hyaline substance. Mitotic figures were frequent in the outer layer of cells, and continued proliferation of these cells occasionally produced papillary extensions into the lumen. More often the proliferating cells seemed to compress the lumens into slitlike spaces that were often crescentic (figs. 10 and 11). A few mitotic figures were also found in the cells next to the lumen (figs. 9 and 10). As the altered cells originating around glandular spaces proliferated, they often formed nests of cells bounded by connective-tissue strands containing abundant collagen (fig. 11). This probably resulted from compression of pre-existent tissue.

With further development of the neoplasm, a more fusiform cell developed from the oval, pale cells, and masses of cells were found that resemble fibroblasts (fig. 12). Fine collagen fibers surrounding these cells could often be demonstrated with van Gieson's stain. The appearance of fusiform cells at a distance from the cuboidal cells suggested that a progressive alteration was taking place. Necrosis was frequent in the center of the epithelial nodules (fig. 12). Some neoplasms closely resem-

bled parotid-gland tumors, and were similar to the tumors previously described (*1*) (fig. 13). Occasional small areas in large tumors somewhat resembled the acinar structure usually found in mammary tumors, but these structures were not lined by single cell layers.

Although the mammary tumors in this group resembled the parotid-gland tumors so closely that it was often difficult to distinguish them, there were some features of the parotid tumors never observed in the mammary tumors. No hyaline material, a characteristic of early stages in the parotid-gland tumors, and no heavy lymphocytic infiltration was ever observed.

Although it has been noted in collections of mammary tumors that morphology has varied somewhat, depending upon the conditions of tumor development such as presence or absence of the milk agent, exposure to chemical carcinogens, hormonal stimulation, and age, the present group is unique in showing such a wide and consistent divergence from the usual groups of mammary tumors. This is further evidence of the alteration in the usual pattern of tumor formation in inbred mice that results from treatment of the newborn with this agent. Rogel and Rudali (*11*), after injecting newborn strain AK mice with cell-free extracts from lymphosarcomas, noted the development of mammary tumors in virgin mice 3 months old. Mammary tumors had not previously been observed in this strain. These authors noted that the tumors differed histologically from mammary tumors in high cancer lines, such as RIII, NLC, or C3H. Two of their photographs resemble our figures 9 and 11.

Another feature of these tumors is the apparent progression from an epithelial cell to a fusiform, fibroblast-like cell. It was previously suggested that the parotid tumors originated from two elements, an epidermal and a mesodermal. Observations on early stages of the mammary tumor, however, suggest a different explanation for the mixed character of these tumors. Progressive modification of the glandular epithelial cells can lead to the development of fusiform cells indistinguishable from fibroblasts.

Hair-Follicle Tumors

These lesions were found in varying numbers and size, but were most frequently multiple, sometimes numbering over a hundred, and often confluent. The lesions were largest in those animals with the greatest numbers of lesions, in general, and when only one or a few lesions were found in a given animal, these rarely measured more than 1 or 2 mm. in diameter. The tumors could be seen and palpated externally in those mice bearing large numbers of the larger lesions, which rarely reached over 1 cm. in diameter. In mice with pigmented hair, even the smallest nodules because of their dense pigmentation, were readily noted on the stretched pelt. In white mice, however, the smaller lesions were difficult to detect, and to find them it was necessary to hold the skin nearly at eye level in a strong light to take advantage of reflected light from the curved surfaces. The lesions tended to be distributed most heavily in the axillary regions

and skin over the shoulder girdle and neck, but were also observed in practically all other areas, including the skin of the legs. None were ever noted in the tail.

Microscopically these tumors appeared to have their origin in the epithelium of the deep portions of the hair follicles, particularly in the bulb. A reconstruction of their development is as follows: The earliest lesion consists of an enlargement and an extension of the bulb deeper into the dermis. At this stage the hair shaft remains within the distal portion of the follicle, though true hair formation at the base has ceased (figs. 18 and 19). Mitoses in the swelling and lengthening bulb are increased, and collections of dendritic melanin-containing cells become prominent at the growing margins, as if attempting to keep pace with the epithelial proliferation. In many of the lesions at this early stage, eosinophilic intranuclear inclusions are found in the proliferative areas. These are identical in appearance to the inclusions previously observed by Stanton (*40*) in similar lesions induced by the agent of Stewart and Eddy. It is interesting that in our material, as in Stanton's, the inclusions were lacking in the larger and presumably older lesions. A more detailed description of the inclusions will be published by Stanton.

As the lesions expand and extend deeper into the dermis, the central portions undergo necrosis, often entrapping nests of the pigmented dendritic cells. The lengthening, tumorous follicle becomes coiled, and the end result is a greatly distended, sac-like structure connected to the follicle of origin, filled with necrotic material incorporating dead pigment cells and small calcific foci (figs. 20 and 21). The periphery of this structure is made up of viable and proliferating epithelium in a stratum of about 10 to 30 cells in thickness. The advancing surface is sharply circumscribed and there is no evidence of infiltration of surrounding tissues. However, the lesions sometimes penetrate the dermis and compress the panniculus carnosus as a result of simple expansion.

Distally, the proliferating activity of follicular epithelium has not been observed to extend beyond the junction of follicle with sebaceous-gland neck (fig. 21). The epidermis has not been involved, and ulceration has not been observed.

Bone Tumors

There is a question as to whether all the lesions designated here as "bone tumors" deserve this label, even under a liberal definition of the term "tumor." The term is used to cover the entire group, nonetheless, because all the lesions appear to represent variants of the same process, which in many instances, at least, was one of definite "new growth."

Basically, these lesions were characterized by proliferation of osteoblasts, with variable degrees of associated osteoid and bone formation. The bones most commonly affected were the vertebrae, cranial bones, ribs, and sternum. In the vertebrae, cranium, and sternum, lesions within the medullary parts of the bone (endosteal) were frequently found

unassociated with either generalized swelling or enlargement of the bone or with any localized exostotic process. In these cases, the medullary cavity was occupied, in varying degree, by new bone formation which sometimes was so extensive as to result in complete ossification of the medullary cavity. In the least extensive examples, the osteoblastic activity resulted only in a thickening of the existing bone trabeculae (fig. 24). This thickening, however, was distinguishable from the underlying normal bone by the larger osteocytes occupying the lacunae of the abnormal bone. Also, these osteocytes tended to exhibit more numerous and more prominent processes than normal osteocytes. The osteocytes in the lesions were more numerous per unit area than osteocytes in adjacent normal bone. As a rule the bone matrix within a lesion appeared less extensively mineralized than normal bone matrix, as judged by a lack of distinct cement lines. There was no significant increase of osteoclasts in these lesions.

In contrast to these endosteal lesions, subperiosteal lesions appeared as definite swellings. In the cranium they formed rounded protuberances that made a depression on the surface of the brain. Occasionally these subperiosteal tumors occurred in the spinal canal as mushroom-shaped growths that compressed the cord and produced paralysis of the hind legs. In the ribs, they grew as fusiform swellings, usually in the proximal half of the rib. Small, nodular, bony enlargements of the ribs at the costovertebral junctions were also found (fig. 23).

Microscopically the subperiosteal proliferations were identical with the endosteal ones already described. Sometimes in the skull subperiosteal lesions were found on both surfaces of the bone, with an endosteal lesion sandwiched between. In these instances it was not possible to determine whether one was dealing with multicentric tumors or with a single tumor that had extended through both tables of a bone.

The majority of bone tumors lacked the usual histologic and cytologic criteria of malignancy, and we have not observed in our own material any distant metastases. Stanton (*40*), however, has noted pulmonary metastases from similar lesions. In our material there were a few examples in which the tumors were microscopically more cellular, contained more frequent mitoses, and displayed much less bone formation than the majority. These appeared to represent cases in which a "higher grade" of the same basic change had taken place.

One bone tumor deserves special comment. It was found in the upper right tibia, adjacent to the knee joint. Microscopically, it was composed of a mixture of cell types. The hematopoietic elements of the medullary cavity of the upper tibia were totally replaced by cells resembling normal adipose cells. However, at the metaphysis, similar adipose cells extended through the cortical bone and were contiguous with a mass of similar cells ensheathing the upper tibia. Within the mass of adipose tissue were small nests of cells, among which osteoid and bone were being formed. The margins of the adipose tissue were composed of flattened cells that appeared to be in the process of differentiating into adipose cells. Thus

within this tumor, differentiation appeared to be directed both toward osseous tissue and toward adipose tissue (fig. 25).

A part of this tumor was transplanted to C3Hf × AKR mice, and has been carried through 21 transfer generations. In the early passages, it continued to produce both fat and bone (fig. 26), but gradually lost both of these signs of functional cells and now resembles an undifferentiated spindle-cell sarcoma. Tissue from the first transfer generation was explanted into tissue culture and has been carried continuously for 16 months (strain GLBT). Even after this period in culture, some cells of adipose type continued to be produced in one tube. When cultures of this cell line were tested for presence of PTA (table 7), negative results were obtained. It appears, therefore, that tumors induced by PTA are not obligatory carriers of it, at least in demonstrable form.

Adrenal Medullary Tumors

Some of these measured up to approximately 1 cm. in diameter, while others could be detected only on microscopic examination. Grossly the cut surface was dark gray and soft. Microscopically, the chromaffin property was generally completely lacking in the tumor cells, and there was little to support the concept of medullary origin except location within the medulla, in the smallest examples. In some of these tumors, certain areas displayed staining and other morphologic properties differing from those of adjacent areas, which suggested multifocal origin followed by different degrees of progression. The tumors grew in a pattern of irregular cords and clumps of cells abundantly supplied with capillaries. In this respect they differed markedly from adrenal medullary tumors in mice not exposed to PTA. Tumors of the latter type have usually shown a distinctive pattern of cords or glandlike formations of low columnar cells.

Renal Tumors

Lesions of the renal-cortical tubules were very commonly observed, as indicated in text-figures 1 and 2. These lesions have been described previously by Stewart *et al.* (*35*). We will therefore note here only that we have not as yet seen any unquestionable tumors originating from this type of lesion. Buffett *et al.* (*12*) have interpreted these lesions as preneoplastic, but justification for this opinion requires that some of the lesions should progress and extend beyond the boundaries of the tubules in which they originated.

A number of spindle-cell tumors have been found in the renal medulla. Occasionally such a tumor filled the renal pelvis and produced hydronephrosis. The cell origin of these tumors is debatable. We have been unable to find any convincing evidence of a possible epithelial origin and currently are inclined to believe they arise from intertubular connective-tissue cells of the medulla. Definite evidence of either smooth or striated muscle cells in these tumors has not been found.

Other Tumors

Tumors of the intraorbital lacrimal glands have been noted and are histologically identical with those of the extraorbital lacrimal glands.

Tumors of the submucosal glands of both serous and mucous type have been commonly found in the nasal passages, the oropharynx, the posterior tongue, and larynx. Histologically these tumors show only slight variations from tumors of the salivary glands, and will not be further described here. In 6 RFM and 2 AKR mice, early proliferations of the surface epithelium of the nasal mucous membrane were seen (fig. 31). Cells in these lesions showed the vacuolated nuclei characteristic of lesions associated with this agent. Lesions at this site have not been found as yet in any other strain.

Small, cellular nodules that appear to represent early tumors of the urethral glands have been noted in 2 male mice. These lesions have a remarkably close resemblance to the early tumors of the salivary glands.

All 5 harderian-gland tumors listed in table 5 were microscopic. The histologic pattern was nearly identical with that of salivary-gland tumors (fig. 22), except that in areas of less extensive alteration there was a papillary pattern.

Tumors of the oral epithelium were found more often in the lower jaw than in the upper. A few displayed some of the characteristics of ameloblastomas, particularly in areas where the epithelium was modified toward a stellate reticulum (fig. 28). Squamous areas were also present in these tumors, which appeared to take origin from the gingival epithelium adjacent to the dental alveoli (fig. 27). Growth was directed along the tooth root, and a grossly visible swelling was sometimes present at the root apex where the tumor expanded. Several mice with these tumors were runts, apparently as a result of difficulty in eating.

Lesions of bronchial epithelium were noted in only 2 mice, one an AKR, the other a C58. These were proliferative lesions of the surface epithelium, but did not invade the bronchial wall (figs. 29 and 30). They were made up of cells with decreased cytoplasm and large, nonpolarized nuclei that often showed the "hollow" character peculiar to early lesions related to PTA. Cell necrosis was also present in these small proliferations.

Metastases

In this work, the only tumors that have been seen to produce metastases were of the parotid and the mammary types. Metastases from these tumors were found only in the lungs—metastases from the mammary tumors became established and grew in the lung, whereas all those that were clearly of parotid origin were contained as emboli within the pulmonary vessels (fig. 32). Occasionally several larger arteries were plugged with tumor, and incomplete infarcts of the parenchyma occurred. In the series of 232 salivary-gland tumors, embolic pulmonary metastases from these tumors were observed 25 times. Free tumor emboli were found twice in the right ventricle of the heart. It is our impression that such

embolic metastases occur more commonly in animals bearing the largest tumors.

Attempts at Transplantation of Tumors and/or Lesions

Our previous attempts to transplant pleomorphic tumors of the parotid gland met with only moderate success (*1*). Where successful serial transfers into recipients of the proper genotype were achieved, a sarcoma-like pattern evolved and predominated. In the present study, 5 of 7 parotid-gland tumors grew progressively and in serial transfer. Three of 4 mammary neoplasms, an adrenal medullary neoplasm, a bone tumor, and a sarcoma of the kidney all grew progressively in appropriate hosts and these were carried serially for at least 2 additional transfers. However, no progressively growing tumors have been observed after transplantation of 14 epitheliomas of the thymus, 4 thyroid tumors, and 2 hair-follicle tumors. Intravenous transfers of thymic tumors in day-old mice have also been unsuccessful.

DISCUSSION

This study has used a number of new methods for the investigation of problems related to the PTA, but these same methods have not been fruitful thus far in disclosing a cell-free, leukemia-inducing agent. This work indicates that there is no positive relationship between PTA and the occurrence of leukemia in mice. It is still possible that a negative or inverse relationship exists, and that PTA may block the manifestation of a leukemia agent.

Even this seems unlikely, judging from the published results of other laboratories [Gross (*41*), Stewart (*3*, *13*), and Latarjet and de Jaco (*9*)], in which increased incidence or an earlier appearance of leukemia, as well as increased incidence of parotid tumors, has been observed after the injection of a single preparation derived from leukemia tissue. Gross (*41*) and Latarjet and de Jaco (*9*) have reported parotid tumors in the same animals in which leukemia was present. We are at a loss, therefore, to explain our failure to alter the incidence of leukemia with any cell-free tissue extract or tissue-culture material that we have tested. We would indeed be hard pressed to select from our data any to indicate a filtrable leukemia-inducing agent. The only observation of possible significance resulted when direct filtrates of L-10813 were used. Leukemias were observed in 4 of 18 C3H × AKR mice at less than 6 months of age (table 3). This is meager evidence for an accelerating factor.

It is possibly relevant that neither tissue cultures nor direct filtrates of L-10813 induced a significant number of parotid tumors, whereas the two other leukemias of C3H/Gs origin ("passage A" series of Gross) showed no leukemia-inducing activity, but abundant parotid tumor-inducing activity. This suggests an inverse relationship between exposure

to A and the incidence of leukemia. A lowered incidence of leukemia in (C3H × AKR)F_1 mice that had received extracts of leukemic tissue was reported by Law, Dunn, and Boyle (*1*), and Buffett *et al.* (*12*) have recently observed a change in this same direction, though the difference was not marked. The evidence is accumulating against the possibility that PTA is also capable of inducing or of accelerating leukemia.

The observation by Rabson and Legallais (*20*), that PTA of a strain developed by Stewart and Eddy, as well as the one developed by us, could produce CPE in strain P388 D_1 has opened new fields for investigation of these agents which are presumably identical or closely related. Rowe *et al.* (*42*) have observed that CF antibody for SE polyoma virus reacts identically with our agent. It should be pointed out that both our material and that of Stewart and Eddy trace back ultimately to AKR leukemic tissue from Gross's laboratory. Both materials have been found to produce CPE in P388 D_1 cultures, and the variety of tumor types induced by them appears to be very similar. Eddy and Stewart (*21*) originally reported propagation of their agent first on monkey kidney and then on normal mouse-embryo cultures (*43*). It would be interesting to know whether propagation of the agent in normal cell systems would result in the emergence of a strain different from one that has been passed through a culture cell strain of leukemic origin, such as P388 D_1.

Concerning the nature of PTA, little can be added from the observations reported here. Filtrability is reconfirmed, and replication of the factor responsible for CPE in tissue culture appears certain when potency is demonstrated, following a dilution factor of 10^{-30} by the tenth passage. These features are consistent with a viral nature of the agent. More convincing of the microbial nature of the agent are the data that account for litter immunity on the basis of maternal infection and antibody development.

We have no information bearing critically on the questions as to whether more than a single agent is involved, whether the agent that produces CPE is also *directly* responsible for the induction of the various tumors, or whether or not the agent may exist in different degrees of biologic or chemical complexity. Our experiments were designed primarily as exploratory moves in search of a leukemia agent and have resulted only in development of methods of investigation of PTA.

The relationship of PTA to leukemia cells *in vivo* needs study. Two of the three leukemic cell strains received from Gross proved to bear the agent, but in one it was not demonstrated. Somewhat equivocal evidence of its presence was obtained for the methylcholanthrene-induced DBA/2 lymphoma, P388. The latter neoplasm deserves much more careful study because of the possibility that the morphologic transformation of P388 lymphoma cells *in vitro* may have been caused or influenced by PTA carried within the tissues *in vivo*. It would be instructive to select transplantable strains of leukemias that do not give evidence of carrying PTA, and to observe the effect of deliberately inoculating them with PTA. It is important to determine whether PTA is contained on or within

leukemia cells themselves or in some stromal cell element such as the histiocyte (macrophage), which is always much in evidence in primary tissue cultures of leukemias.

The possibility of natural transmission of PTA from mouse to mouse within a colony complicates all experiments in this field. It must be considered in all experimental planning and in the interpretation of the results. During our observation we have not seen an animal develop parotid tumors unless it had received some type of material by injection. However, the development of parotid tumors in 2 of 64 mice that, as controls, received tissue-culture medium alone should be explained. This result might be obtained if the agent were present in the medium LN40, or it might result if PTA were present on the skin surface and introduced into the subcutaneous tissues by the needle. It might also be the result of activation of latent agent by nonspecific effects of the inoculum (foreign protein) or the injection procedure (traumatic stress). Woolley's (*8*) observation that parotid tumors could be induced by cortisone treatment of certain strains of mice supports either the possibility that a stress reaction facilitates the tumor-inducing effect or the concept that parotid tumors of mice can be induced by more than one factor (virus, steroid). Because of these complications it will be necessary, in future work, to have sensitive methods for detection of the latent agent in test animals, as well as for prevention of exposure of test animals to the agent by whatever routes it may travel (air-borne particles, food, bedding, insect vectors, etc.). Information in this regard has recently been obtained by Rowe *et al.* (*26*) using the complement-fixation test (*42*) and hemagglutination inhibition reaction (*44*) in a survey of animals of several mouse colonies.

Whatever the physical and biochemical properties of PTA eventually prove to be, observations of a biologic type concerning the interaction of host and agent have established certain cardinal facts that will be of value in explaining the mechanism of tumorigenesis when this agent is involved. These facts, taken from work of others as well as our own, may be listed as follows:

1. The importance of age to host susceptibility. The optimal age of susceptibility is still unknown and may even lie in the prenatal period. However, it is established that susceptibility to tumor induction diminishes progressively after birth and virtually disappears at or before weaning, if the subcutaneous route of inoculation is used in otherwise untreated mice. It appears that total-body X irradiation can modify "age" resistance, and it is necessary now to determine whether X irradiation exerts its effect through impairment of immune responses or through some direct effect on the cells that grow into tumors.

2. The multicentric origin of tumors within a given organ.

3. The selection of a limited group of tissue sites in a given species. In the mouse these are the salivary glands, extraorbital lacrimal glands, accessory salivary glands of oronasopharynx, submucosal glands of upper respiratory tract, thymus, thyroid, breast, hair follicles, adrenal medulla,

renal pelvic connective tissues, bones, and subcutaneous connective tissues. Although it is true that this is an ostensibly diverse array, and that occasional additional sites have shown involvement, tumors are generally limited to these sites. It is stressed also that tumors of other sites are relatively rare in comparison with the incidence of salivary-gland tumors and, with certain preparations, of thymic epitheliomas. Such tissues as the central and peripheral nervous system, epidermis, blood-forming elements, pancreas, liver, genital tract, skeletal, cardiac, smooth muscle, and epithelium of the alimentary tract generally appear exempt.

At the present time, one should sensibly be reluctant to choose a "common denominator" of the various organs in the mouse that have proved susceptible to this agent. However, the majority of the tissues that develop tumors have the common characteristic that they are incompletely developed at birth and must continue to proliferate and to differentiate for some time afterward. Thus, most of the organs involved fall into the class of "appendages" or terminal derivatives of major systems: The mammary glands and hair follicles are appendages of the epidermis; the salivary glands, submucosal glands of oronasopharynx, odontogenic epithelium, the thyroid gland, and the thymus are appendages of the alimentary tract. To use a metaphor somewhat freely, PTA appears to produce a disease of certain twigs and leaves of the ontogenetic tree, rather than of the trunk and major branches. One is tempted to wonder whether the analogy may apply to similarities between "susceptibility" to the agent and "competence" to respond to morphogenetic evocators. Some explanation is needed to account for the refractoriness of such "twigs and leaves" as the parathyroid gland, liver, and pancreas, to mention only a few exceptions to the proposed rule.

4. The susceptibility of several species [mouse, rat (*45*), hamster (*46*)] to the agent, and the occurrence of a different range of characteristic tumor types in each species. It seems likely that a definite range of species and organ susceptibility will eventually be delineated.

5. The histologic characteristics and the sequence of cytologic changes during the development of tumors at several different sites are similar in the mouse. Thus, the first changes in the salivary glands, submucosal glands of upper respiratory tract, and thyroid are the loss of the specific differentiative characteristics of the cytoplasm of the cells, with transformation to a less clearly differentiated form. Since this initial change appears to progress at different rates and in varying degrees at different foci within a given organ, it leads to a wide range of states of cell transformation that altogether gives the pleomorphic picture characteristic of these tumors.

REFERENCES

(*1*) Law, L. W., Dunn, T. B., and Boyle, P. J.: Neoplasms in the C3H strain and in F_1 hybrid mice of two crosses following introduction of extracts and filtrates of leukemic tissues. J. Nat. Cancer Inst. 16: 495–539, 1955.

(2) GROSS, L.: A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc. Soc. Exper. Biol. & Med. 83: 414–421, 1953.

(3) STEWART, S. E.: Leukemia in mice produced by a filterable agent present in AKR leukemic tissues with notes on a sarcoma produced by the same agent. Anat. Rec. 117: 532, 1953.

(4) ———: Neoplasms in mice inoculated with cell-free extracts or filtrates of leukemic mouse tissues. I. Neoplasms of the parotid and adrenal glands. J. Nat. Cancer Inst. 15: 1391–1415, 1955.

(5) GROSS, L.: "Spontaneous" leukemia developing in C3H mice following inoculation, in infancy, with Ak-leukemic extracts, or Ak-embryos. Proc. Soc. Exper. Biol. & Med. 76: 27–32, 1951.

(6) ———: Studies on the nature and biological properties of a transmissible agent causing leukemia following inoculation into newborn mice. Ann. New York Acad. Sc. 68: 501–521, 1957.

(7) DULANEY, A. D.: Parotid gland tumor in AKR mice inoculated when newborn with all-free Ak leukemic extracts. Cancer Res. 16: 877–879, 1956.

(8) WOOLLEY, G. W., and SMALL, M. C.: Strain specificities of leukemia agent. Ann. New York Acad. Sc. 68: 533–539, 1957.

(9) LATARJET, R., and DE JACO, M.: Production de cancers multipler chez des souris AKR agent recu un extract leucemique a-cellulaire isologue. Compt. rend. Acad. sc. 246: 499–501, 1958.

(10) LATARJET, R., REBEYROTH, N., and MOUSTACCHI, E.: Production de cancers multipler chez des souris, avant recu de l'acide nucleique extrait de tissue leucemiques isologues un homologues. Compt. rend. Acad. sc. 246: 853-855, 1958.

(11) ROGEL, R., and RUDALI, G.: Production de tumeurs epitheliales chez les souris a l'aide d'extraits a-cellulaires saccharosignes de lymphosarcomes. Bull. du Cancer 44: 483–492, 1957.

(12) BUFFETT, R. F., COMMERFORD, S. L., FURTH, J., and HUNTER, M. J.: Agent in Ak leukemic tissues, not sedimented at 105,000 g, causing neoplastic and non-neoplastic lesions. Proc. Soc. Exper. Biol. & Med. 99: 401–407, 1958.

(13) STEWART, S. E.: Neoplasms in mice inoculated with cell-free extracts or filtrates of leukemic mouse tissues. II. Leukemia in hybrid mice produced by cell-free filtrates. J. Nat. Cancer Inst. 16: 41–53, 1955.

(14) WOOLLEY, G. W., and SMALL, M. C.: Experiments on cell-free transmission of mouse leukemia. Cancer 9: 1102–1106, 1956.

(15) DULANEY, A. D., MAXEY, M., SCHILLIG, M. G., and GOSS, M. F.: Neoplasms in C3H mice which received Ak-leukemia extracts when newborn. Cancer Res. 17: 809–814, 1957.

(16) RUDALI, G., DUPLAN, J. F., and LATARJET, R.: Latence des leucoses chez des souris Ak injectees avec un extrait leucémique a-cellulaire Ak. Compt. rend. Acad. sc. 242: 837–839, 1956.

(17) HAYES, E. F., SIMMONS, N. S., and BECK, W. S.: Induction of mouse leukemia with purified nucleic acid preparations. Nature, London 180: 1419–1420, 1957.

(18) ROWE, W. P., and others: Isolation of cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exper. Biol. & Med. 84: 570–573, 1953.

(19) DAWE, C. J., and POTTER, M.: Morphologic and biologic progression of a lymphoid neoplasm of the mouse in vivo and in vitro. Am. J. Path. 33: 603, 1957.

(20) RABSON, A. S., and LEGALLAIS, F. Y.: Cytopathogenic effect produced by polyoma virus in cultures of milk-adapted lymphoma cells (strain P388 D_1). Proc. Soc. Exper. Biol. & Med. 100: 229–233, 1959.

(21) EDDY, B. E., STEWART, S. E., and BERKELEY, W.: Cytopathogenicity in tissue cultures by a tumor virus from mice. Proc. Soc. Exper. Biol. & Med. 98: 848–851, 1958.

(*22*) DUNN, T. B.: Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J. Nat. Cancer Inst. 14: 1281–1433, 1954.

(*23*) POTTER, M.: Personal communication.

(*24*) GROSS, L.: Development and serial cell-free passage of a highly potent strain of mouse leukemia virus. Proc. Soc. Exper. Biol. & Med. 94: 767–771, 1957.

(*25*) DAWE, C. J., POTTER, M., and LEIGHTON, J.: Progressions of a reticulum-cell sarcoma of the mouse *in vivo* and *in vitro*. J. Nat. Cancer Inst. 21: 753–781, 1958.

(*26*) ROWE, W. P., HARTLEY, J. W., LAW, L. W., and HUEBNER, R. J.: Studies of mouse polyoma virus infection. III. Distribution of antibodies in laboratory mouse colonies. J. Exper. Med. 109: 449–462, 1959.

(*27*) MIRAND, E. A., MOUNT, D. T., MOORE, G. E., GRACE, J. T., JR., and SOKAL, J. E.: Induction of tumors by a virus-like agent(s) released by tissue culture. Proc. Soc. Exper. Biol. & Med. 99: 1–5, 1958.

(*28*) GROSS, L.: Transmission of Ak leukemic agent into newborn mice of the C57 brown/cd inbred line. Proc. Soc. Exper. Biol. & Med. 86: 734–739, 1954.

(*29*) ———: Leukemic filtrates inducing leukemia or parotid tumors after inoculation into newborn mice less than 16 hours old, and leukemia only when inoculated into suckling mice 1–14 days old. Proc. Am. Assoc. Cancer Res. 2: 304, 1958.

(*30*) DEBRUYN, W. M.: In vitro cultivation of malignant lymphoblasts of transplantable mouse lymphosarcoma MB(T86157) without typical mesenchyme cells. Bijdr. Dierk. 28: 77–85, 1949.

(*31*) RABSON, A. S.: Personal communication.

(*32*) TAKEMOTO, K. K., and HABEL, K.: Virus-cell relationship in a carrier culture of HeLa cells and Coxsackie A9 virus. Virology 7: 28–44, 1959.

(*33*) RUDALI, G., DUPLAN, J. F., and LATARJET, R.: Leucemogenese chez des souris AKR injectees a la naissance avec un extrait leucemique aqueux isologue. Bull. du Cancer 44: 440–443, 1957.

(*34*) LAW, L. W., ROWE, W. P., and HARTLEY, J. W.: The relationship of parotid tumor (polyoma) agent and lymphocytic neoplasms of the mouse. In preparation.

(*35*) STEWART, S. E., EDDY, R. E., GOCHENOUR, A. M., GORGEN, N. G., and GRUBBS, G. E.: The induction of neoplasms with a substance released from mouse tumor by tissue culture. Virology 3: 380–400, 1957.

(*36*) STEWART, S. E., EDDY, B. E., and BORGESE, N.: Neoplasms in mice inoculated with a tumor agent carried in tissue culture. J. Nat. Cancer Inst. 20: 1223–1243, 1958.

(*37*) CASTLEMAN, B.: Tumors of the thymus gland. Atlas of Tumor Pathology, Section V-Fascicle 19. Washington, D.C., Armed Forces Inst. of Pathology, 1955, p. 8.

(*38*) ANDERVONT, H. B., DUNN, T. B., and CANTER, H. Y.: Susceptibility of agent-free inbred mice and their F_1 hybrids to estrogen-induced mammary tumors. J. Nat. Cancer Inst. 21: 783–811, 1958.

(*39*) FURTADO-DIAS, M. TH.: Lesions testiculaire et adenocarcinome spontane de la mamelle chez les souris de la lignee He. Origine de la lignee He. Arq. Pat. 22: 404–411, 1950.

(*40*) STANTON, M. F.: Personal communication.

(*41*) GROSS, L.: Filterable agent causing leukemia following inoculation in mice. Texas Rep. Biol. & Med. 15: 603–626, 1957.

(*42*) ROWE, W. P., HARTLEY, J. W., BRODSKY, I., and HUEBNER, R. J.: Complement fixation with a mouse tumor virus (S.E. polyoma). Science 128: 1339–1340, 1958.

(*43*) EDDY, B. E., STEWART, S. E., and GRUBBS, G. E.: Influence of tissue culture passage, storage, temperature and drying on viability of SE polyoma virus. Proc. Soc. Exper. Biol. & Med. 99: 289–292, 1958.

(44) EDDY, B. E., ROWE, W. P., HARTLEY, J. W., STEWART, S. E., and HUEBNER, R. J.: Hemagglutination with the SE polyoma virus. Virology 6: 290–291, 1958.

(45) EDDY, B. E., STEWART, S. E., STANTON, M. F., and MARCOTTE, J. M.: Induction of tumors in rats by tissue-culture preparation of SE polyoma virus. J. Nat. Cancer Inst. 22: 161–171, 1959.

(46) EDDY, B. E., STEWART, S. E., YOUNG, R., and MIDER, G. B.: Neoplasms in hamsters induced by mouse tumor agent passed in tissue culture. J. Nat. Cancer Inst. 20: 747–761, 1958.

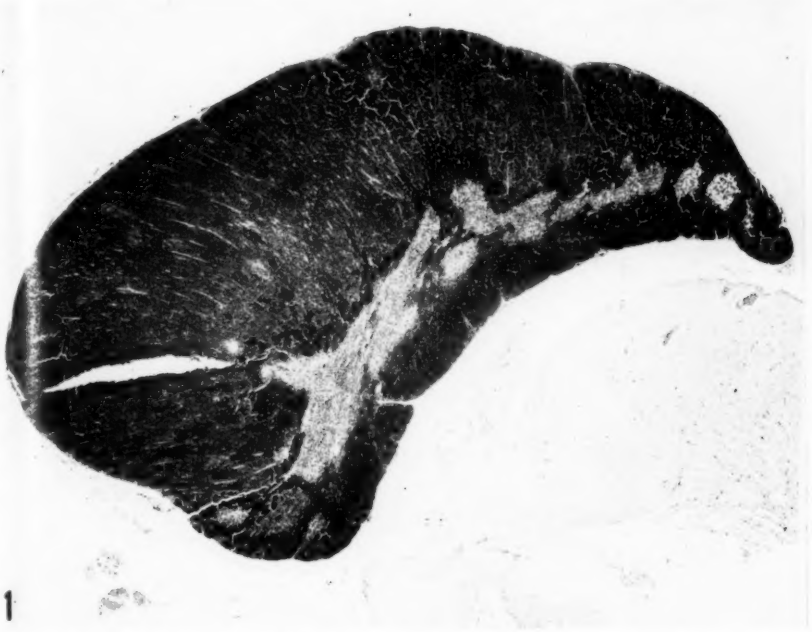
PLATES

511629—59——13

PLATE 51

FIGURE 1.—Section of thymus gland from 21-day-old (C3Hf × AKR)F_1 mouse inoculated at birth with first passage of PTA in P388 D_1 culture. The ventral cortex (*above* and to *left* of pale medulla) is hyperplastic and less intensely stained because of diffuse proliferation of thymic epithelium. (*See also* fig. 3.) × 24

FIGURE 2.—Focal solitary epithelial tumor in thymus of a 4-month-old (C3Hf × AKR)F_1 mouse that received material from short-term culture of leukemia from Gross's induction experiments ($GLTC_1$; *see* table 2). Necrosis is present in *center* of tumor. This lesion was not detected grossly. × 69



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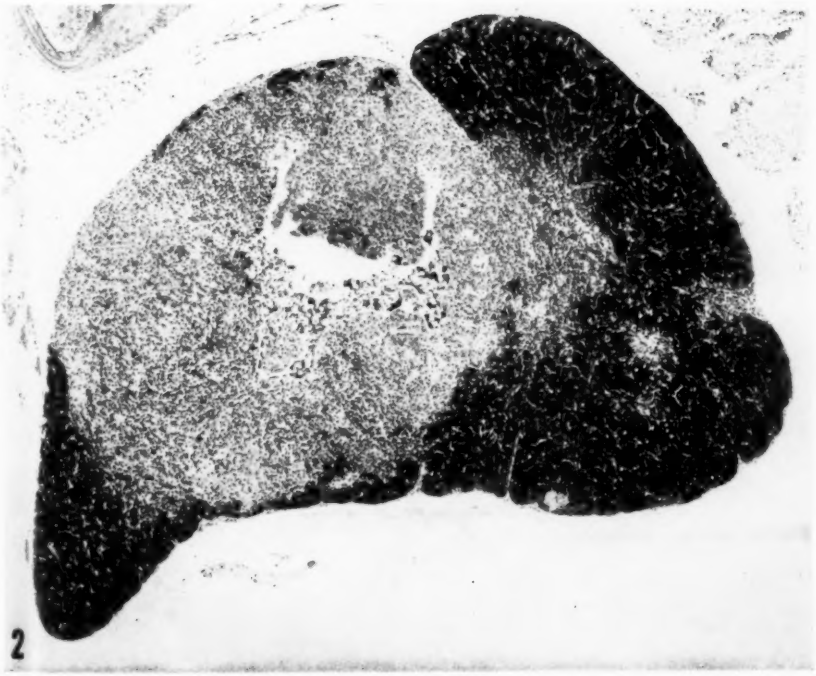

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PLATE 52

FIGURE 3.—Section of thymic cortex from same animal shown in figure 1. *Note* closely packed proliferating thymic epithelium (pale cells) immediately beneath capsule and along capillaries perpendicular to capsule. This represents the earliest detectable stage in development of the diffuse type of thymic epithelioma. × 565

FIGURE 4.—Section of large thymic epithelial tumor in 4-month-old (C3Hf × AKR)F_1 mouse that received material from short-term culture of leukemia of Gross's induction experiments ($GLTC_1$; *see* table 2). Tumor has invaded beyond the thymic capsule, which is still recognizable, running obliquely through *center* of field. Cells here are round or spindled and have little resemblance to epithelium. × 520

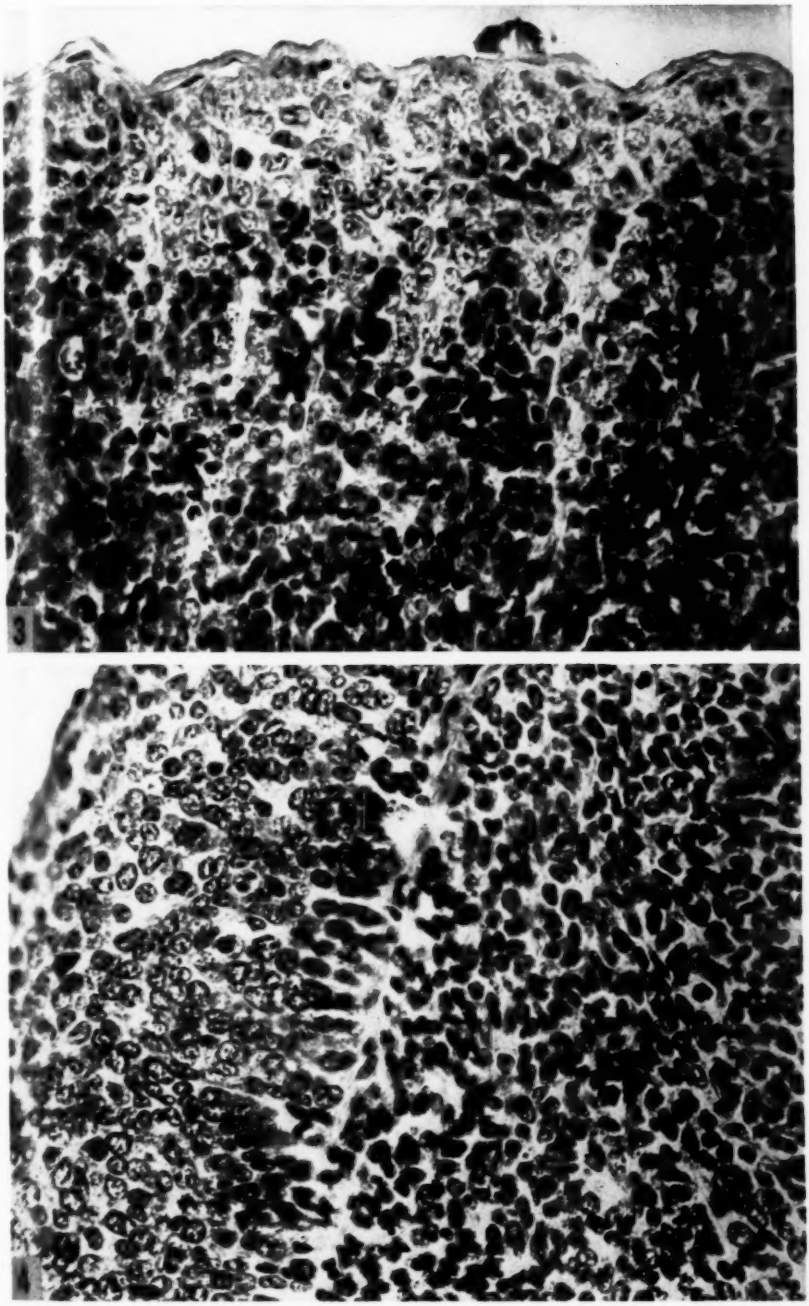


PLATE 53

FIGURE 5.—Section of another area of same tumor shown in figure 4. *Note* epithelial characteristics of cells palisaded around blood vessels. × 850

FIGURE 6.—Section from same tumor shown in figures 4 and 5. Palisaded epithelium is separated from capillary wall by weakly staining fluid that is not visible here. Cells away from vessels show lack of epithelial characteristics. *Note* similarity of histologic picture shown in figures 5 and 6 to that of human thymic tumors illustrated in Castleman's figure 34 and plate III-A (*37*).

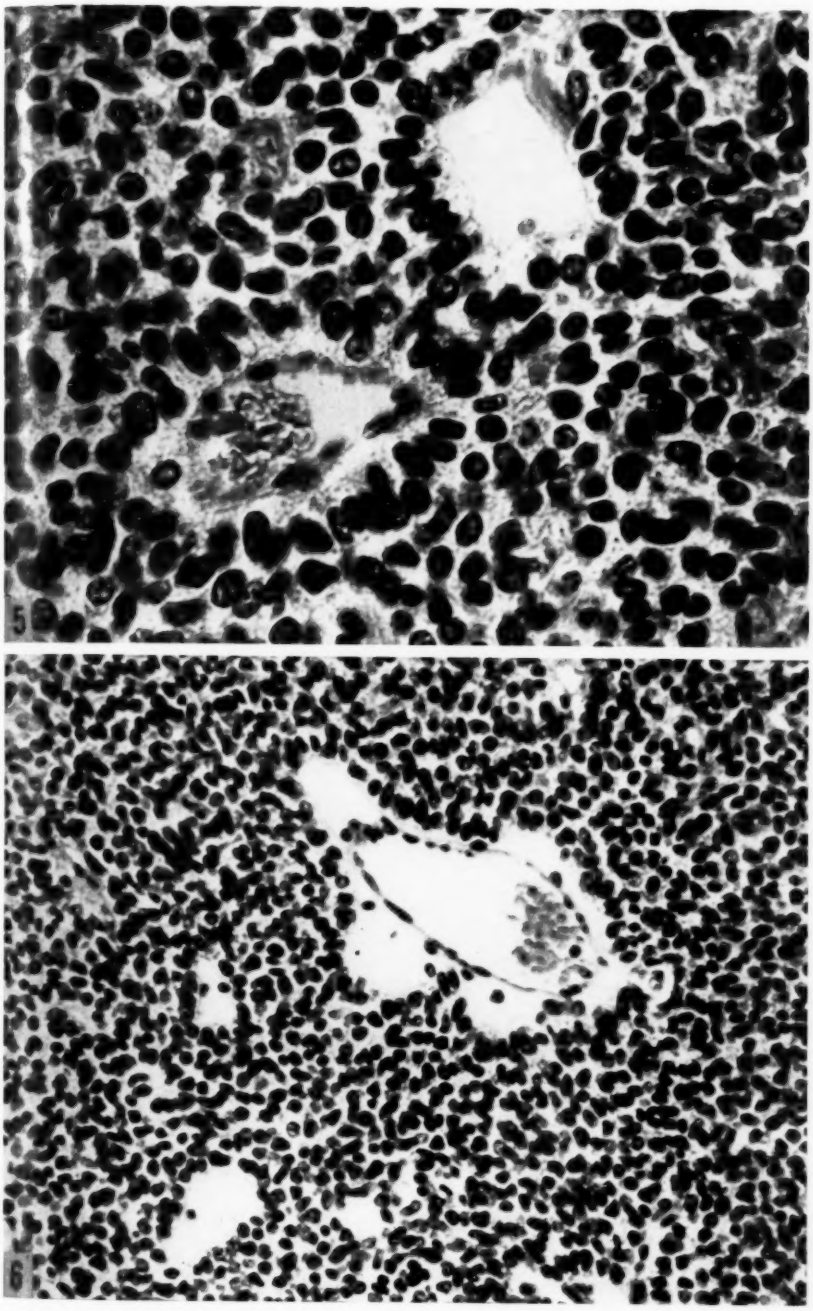
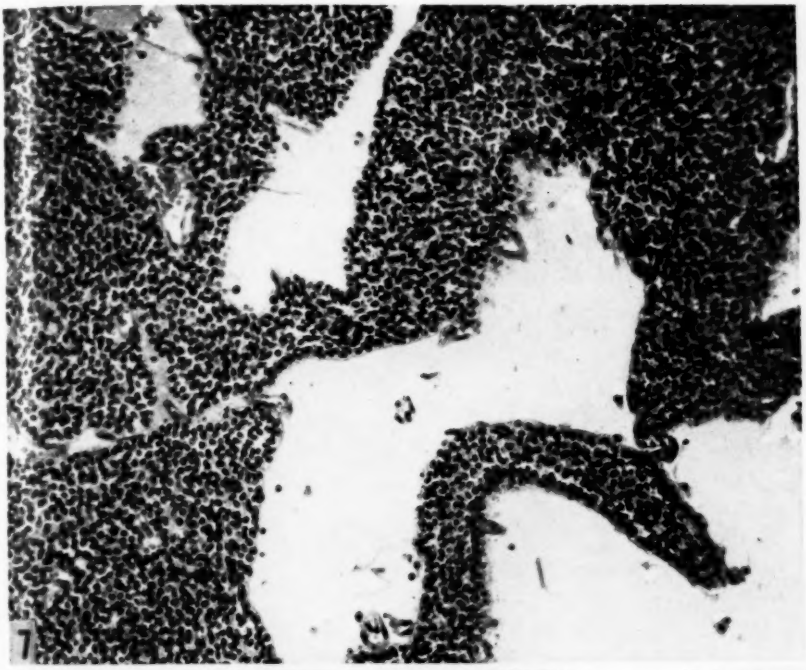


PLATE 54

FIGURE 7.—Same thymic epithelioma shown in figure 6, with more extensive collections of fluid between tumor and vessels, and formation of large "lakes." × 400

FIGURE 8.—Higher magnification of an area from figure 7. *Note* epithelial character of cells at interface between tumor and coagulated fluid. A capillary is present between the two cell masses. × 650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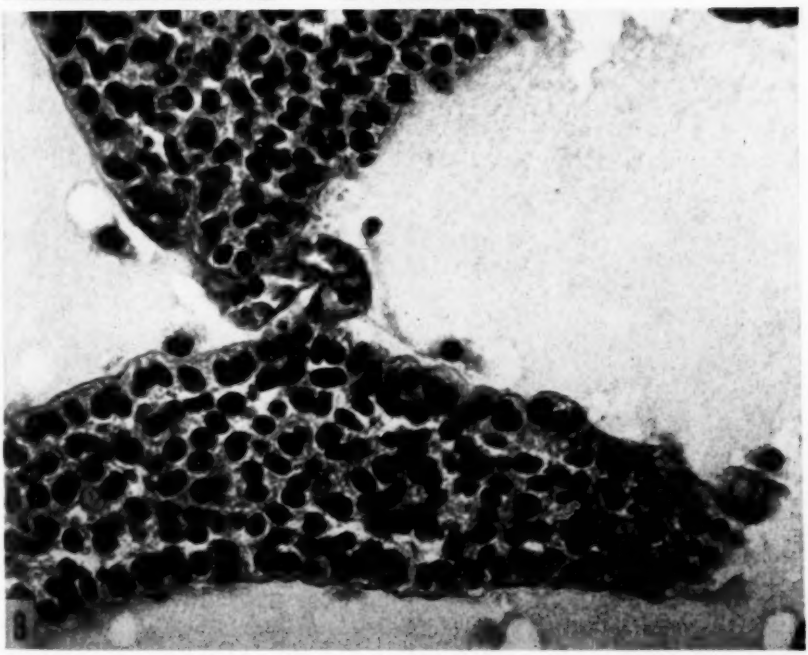
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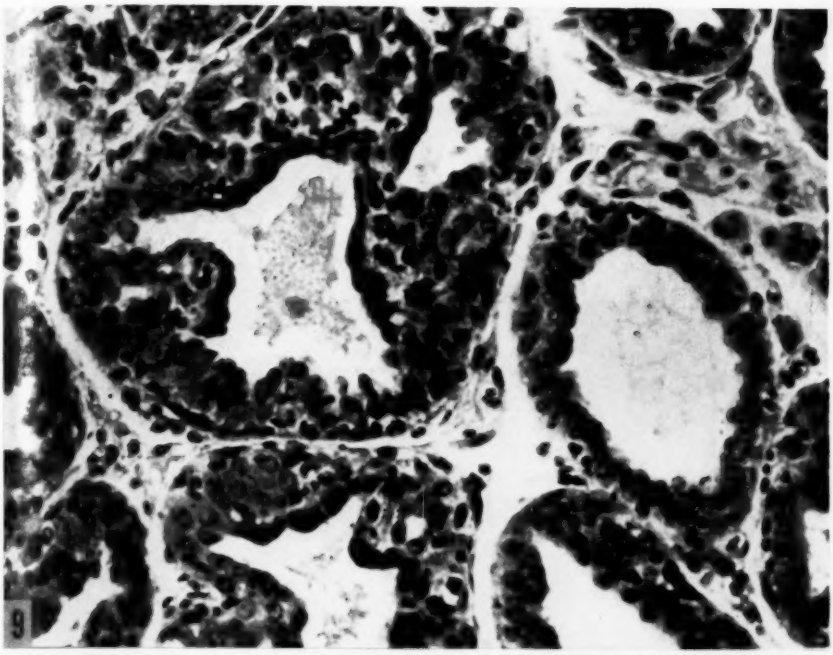
PLATE 55

FIGURE 9.—(Strain C3Hf/Bi female, age 2½ months, inoculated when newborn with first passage of PTA in P388 D_1 culture.) Mammary gland, early neoplastic change. *Note* dilated duct-like spaces lined by an inner layer of basophilic cuboidal cells and outer layers of paler, more oval cells. Mitotic figures are seen in both inner and outer layers, but proliferation is more active in outer layers, with resultant narrowing of luminal spaces. Hematoxylin and eosin. × 400

FIGURE 10.—(Strain C3Hf/Bi female, age 2½ months, inoculated when newborn with first passage of PTA in P388 D_1 culture.) Proliferating cells in duct-like spaces. *Note* papillary projection into lumen. At *top right* is the margin of a mass of neoplastic cells with a crescentic space at *left* margin. × 210

FIGURE 11.—Another area from same tumor. *Note* masses of neoplastic cells, probably a further development from duct-like structures. *Note* crescentic spaces lined by more basophilic cuboidal cells. × 135



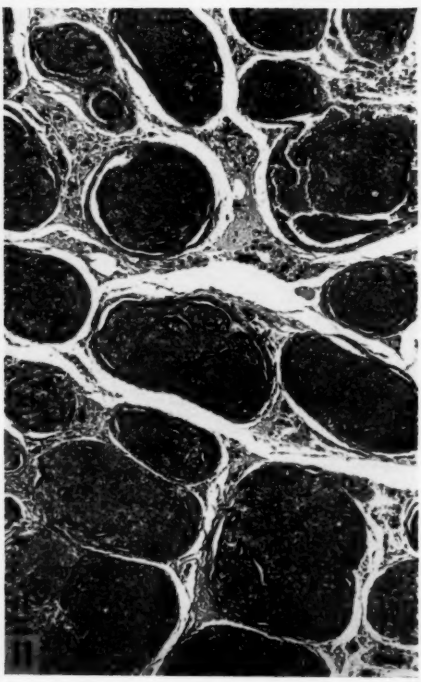
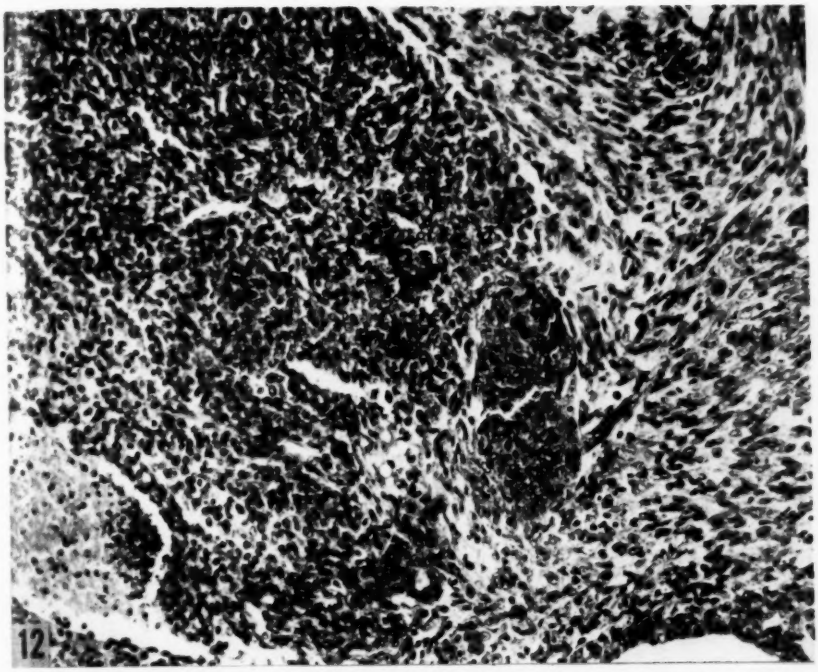


PLATE 56

FIGURE 12.—(Strain C3Hf/Bi female, age 2 months, inoculated when newborn with first passage of PTA in P388 D_1 culture.) Area from large mammary tumor showing necrosis in *lower left*, epithelial cells in *center*, and pale fusiform cells on *right*. × 210

FIGURE 13.—(Strain C3Hf/Bi male, age 7 months, inoculated when newborn with short-term culture from leukemia of Gross ($GLTC_1$).) Area from large mammary tumor showing resemblance to parotid-gland tumors. *Note* space lined by cuboidal cells and pale fusiform cells between. × 400



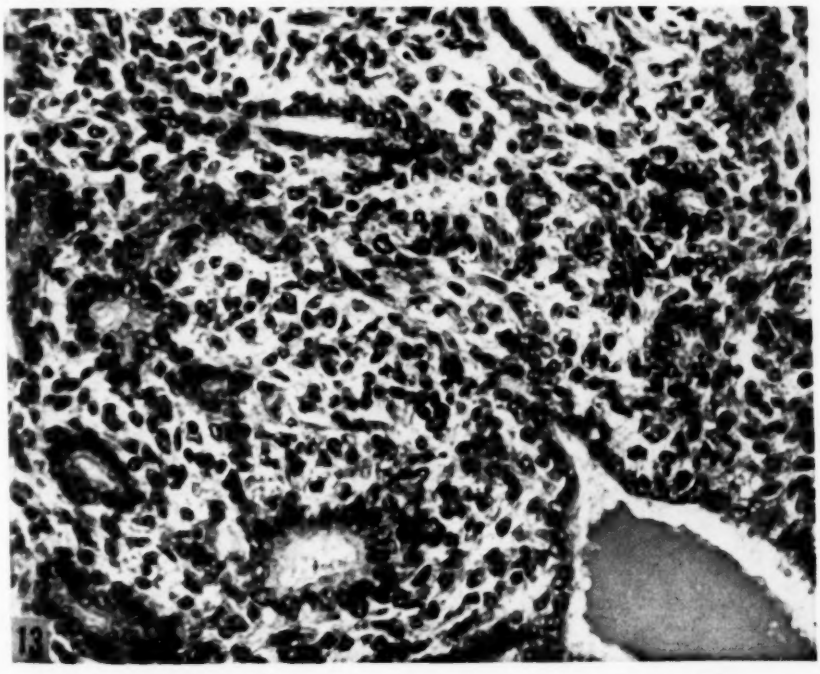
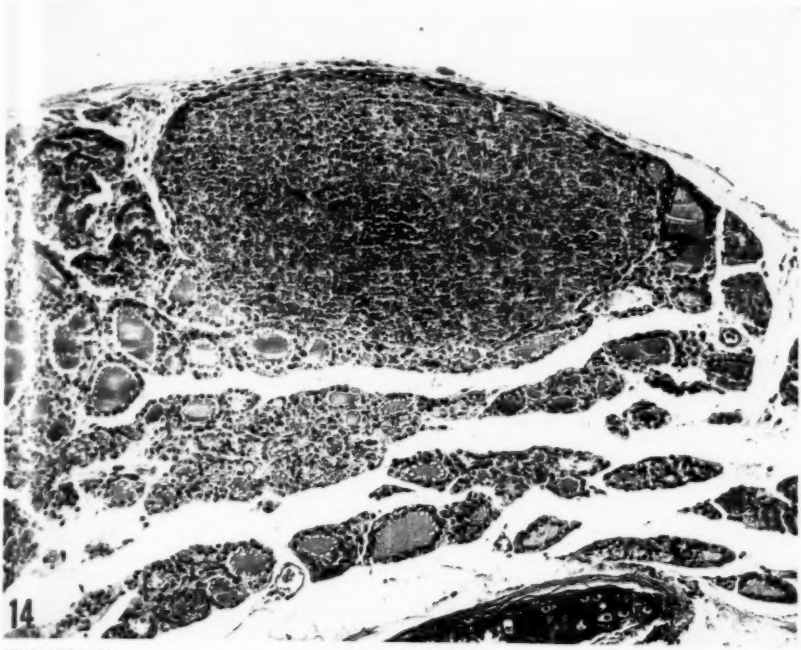


PLATE 57

FIGURE 14.—Thymic epithelial tumor in an ectopic focus of thymic tissue adjacent to a parathyroid gland (*left*), and within the thyroid capsule. This animal also had a thymic tumor involving the intrathoracic thymus. × 135

FIGURE 15.—Two small thyroid tumors, one in each lobe of the thyroid gland of a 3-month-old C3Hf/Bi mouse that had received the first passage of PTA in P388 D_1 cultures. *Note* presence of colloid within tumor on *left*. × 50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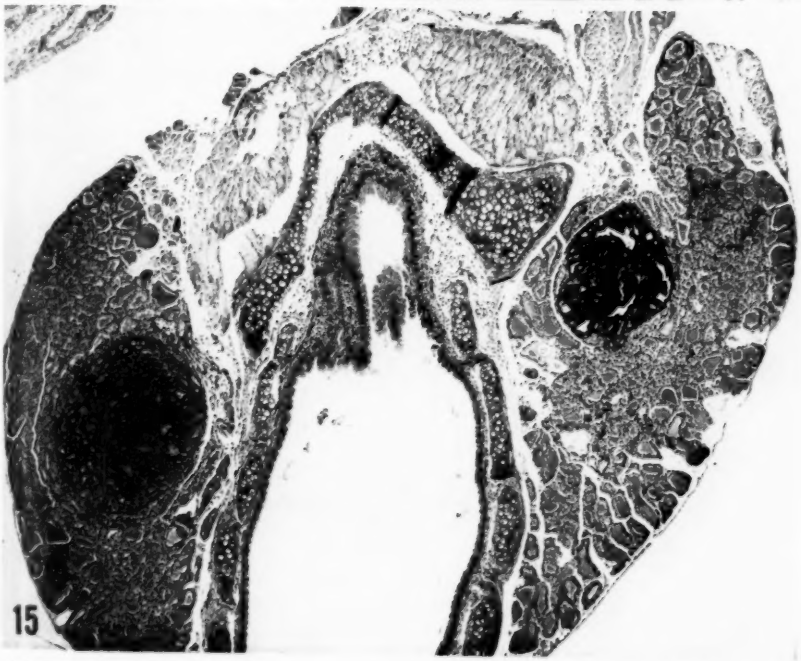
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PLATE 58

FIGURE 16.—Thyroid containing multiple early lesions in a 2-month-old (C3H × AKR)F_1 mouse that had received PTA after first passage in P388 D_1 culture. Dark areas in *upper right* contain many lymphocytes and plasma cells not identifiable at this magnification. × 85

FIGURE 17.—Higher magnification of portion of field shown in figure 16. Some cells of this follicle (*upper right*) show no alteration, while cells on *left* have enlarged nuclei and are proliferating to form a mass that displaces colloid. *Note* some nuclei with large nucleoli and others with large clear, vacuole-like areas. × 690

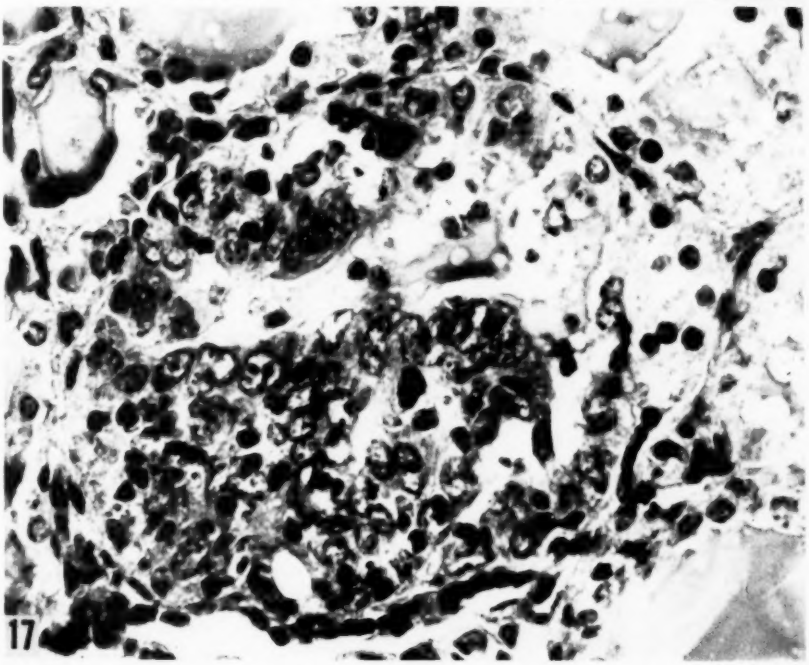

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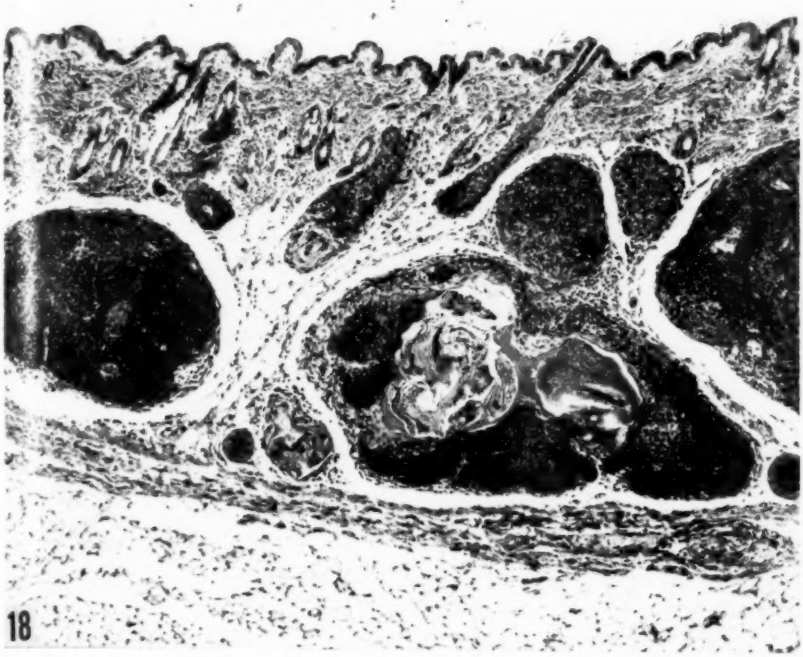
PLATE 59

FIGURE 18.—Tumors of hair follicles in 3-month-old (C3Hf × AKR)F_1 mouse that received at birth PTA from first passage in P388 D_1 cultures. *Note* tumors of varying size and stage of development from hair follicles. Panniculus carnosus is not invaded. × 85

FIGURE 19.—Higher magnification of follicle included in figure 18. *Note* retention of hair shaft in this relatively early lesion. Mitoses are visible among proliferating cells of the bulb. × 260



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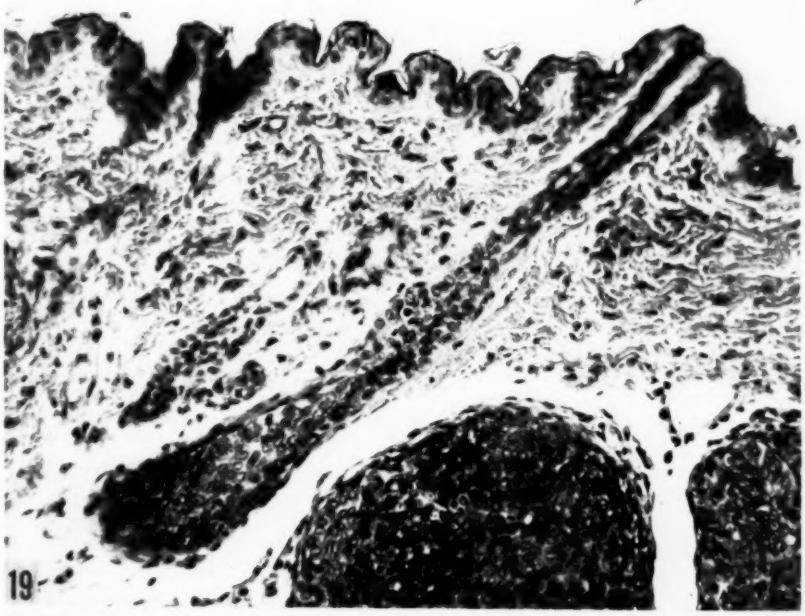

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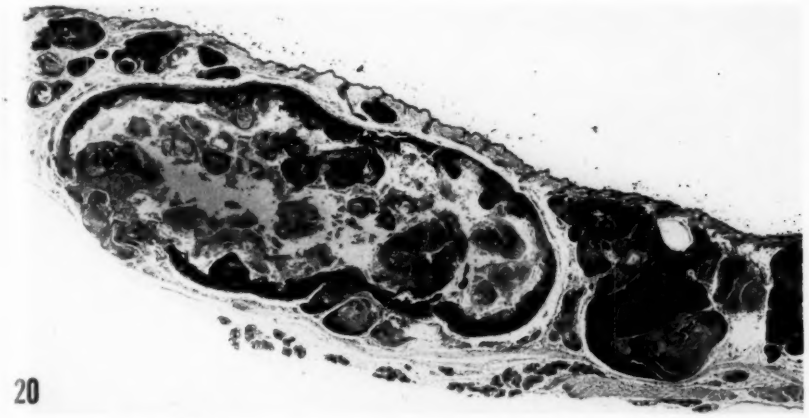
PLATE 60

FIGURE 20.—Advanced hair-follicle tumors in a 2-month-old C3Hf/Bi mouse. Central portion of largest tumor is completely necrotic, as well as peripheral portion at left. Underlying mammary tissue is not invaded. × 15

FIGURE 21.—Higher magnification of tumor shown in figure 20. *Note* limitation of proliferative process to follicle below neck of sebaceous gland. × 200

FIGURE 22.—Low magnification of tumor of harderian gland in a 2-month-old C58 mouse inoculated at birth with PTA after first passage through P388 D_1. Cystic areas within tumor are similar to those often present in extraorbital lacrimal-gland tumors. × 90



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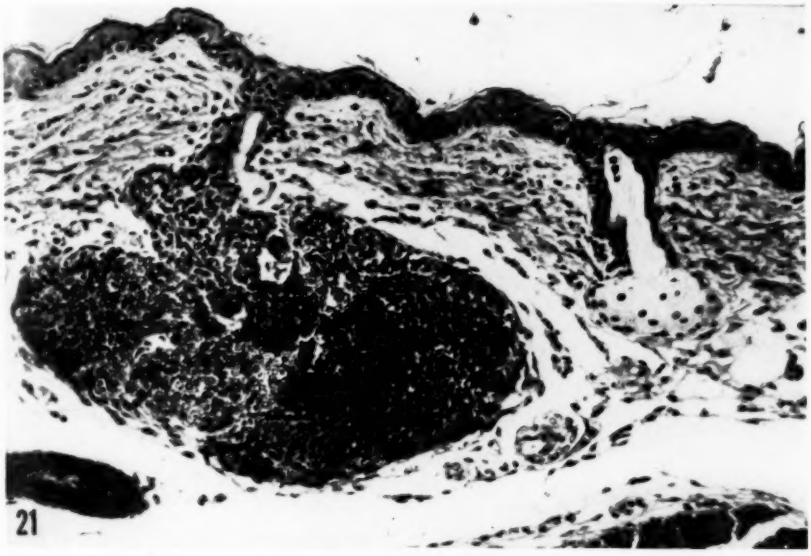

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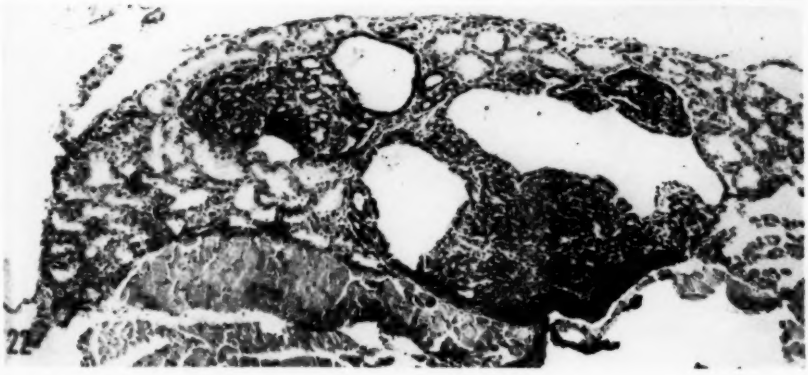

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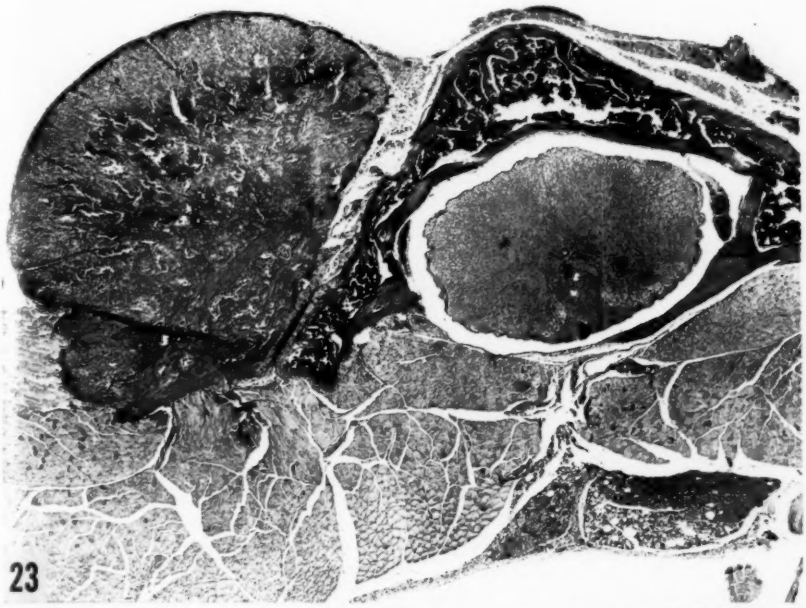
PLATE 61

FIGURE 23.—Bone tumor in a 3.5-month-old C3Hf/Bi mouse that received at birth material from short-term leukemia culture ($GLTC_1$). Tumor arises from a rib near its articulation with the transverse process of a thoracic vertebra. *Note* peripheral cellular proliferation with central osteoid and bone formation. × 35

FIGURE 24.—Bone lesions in vertebral body of a 3-month-old C3Hf/Bi mouse that received at birth material from short-term leukemia culture (L-12289). Lesion is chiefly endosteal, though there is also subperiosteal activity at the *left*. New osteoid is being laid down around pre-existing normal bone trabeculum (*right*) and on both sides of cortical plate (*left*). × 430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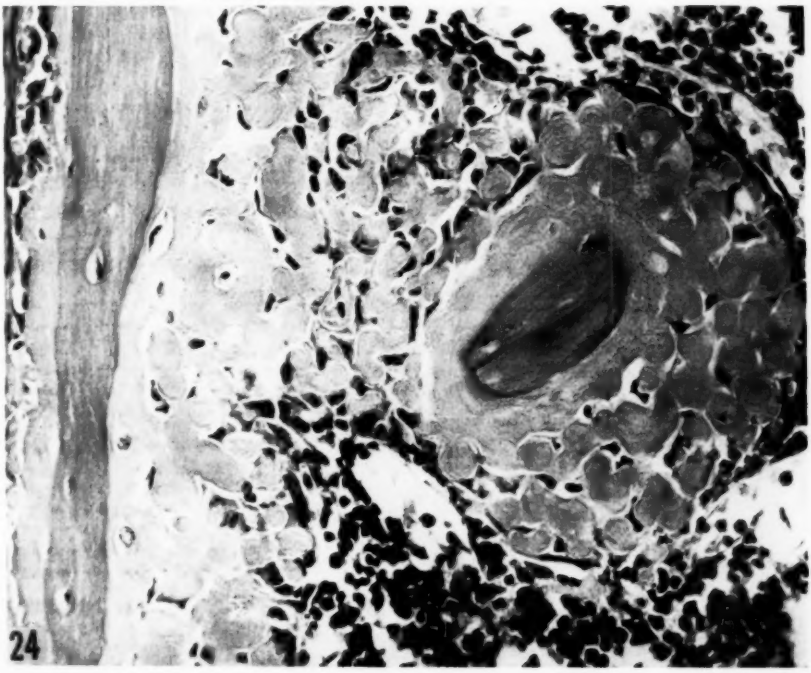
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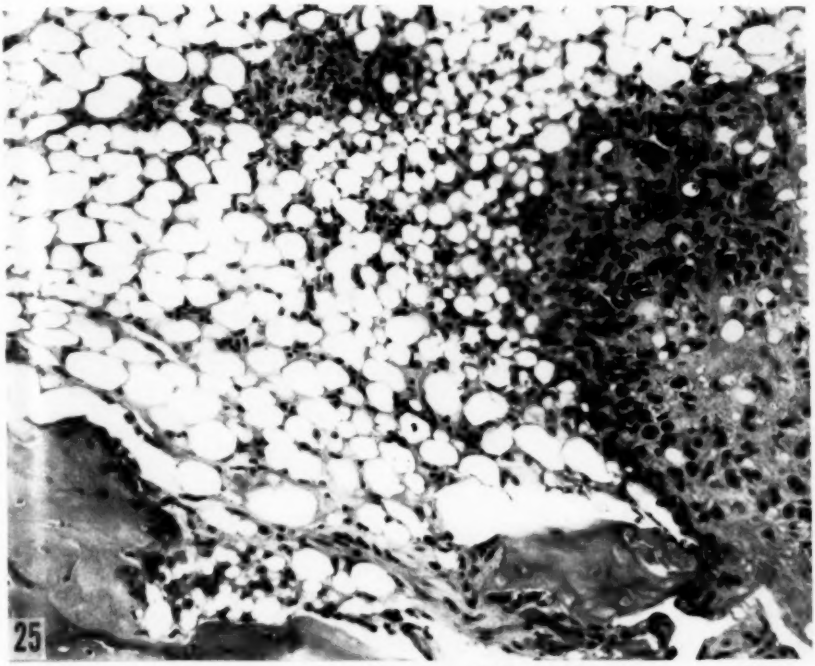
PLATE 62

FIGURE 25.—Primary mixed tumor of fat and bone in medullary cavity of tibia of 4-month-old (C3Hf × AKR)F_1 mouse. Animal had received at birth filtrate of short-term culture of leukemia ($GLTC_1$). × 210

FIGURE 26.—First transfer generation of tumor shown in figure 24. Differentiation of proliferating cells into adipose cells peripherally and osteocytes centrally is more clearly evident here than in the primary. × 55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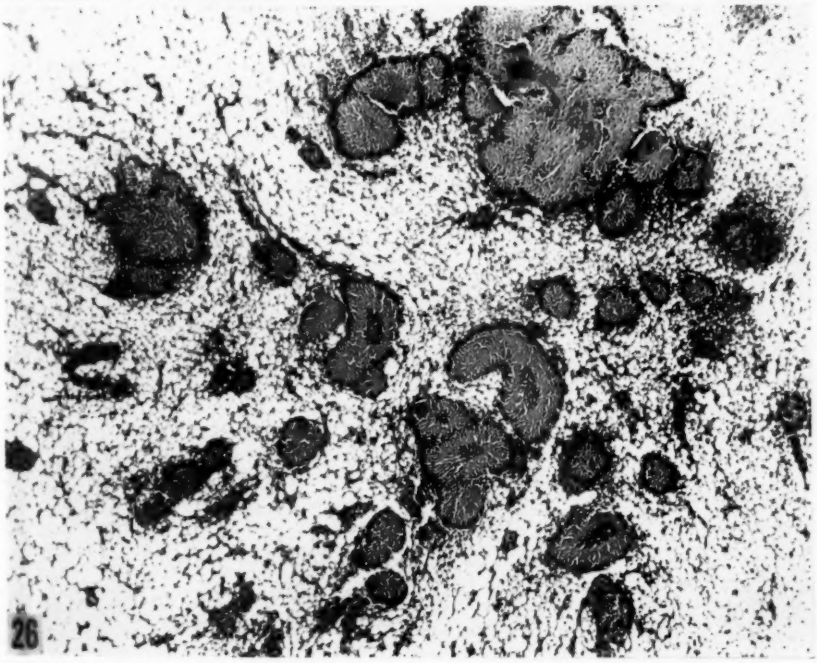
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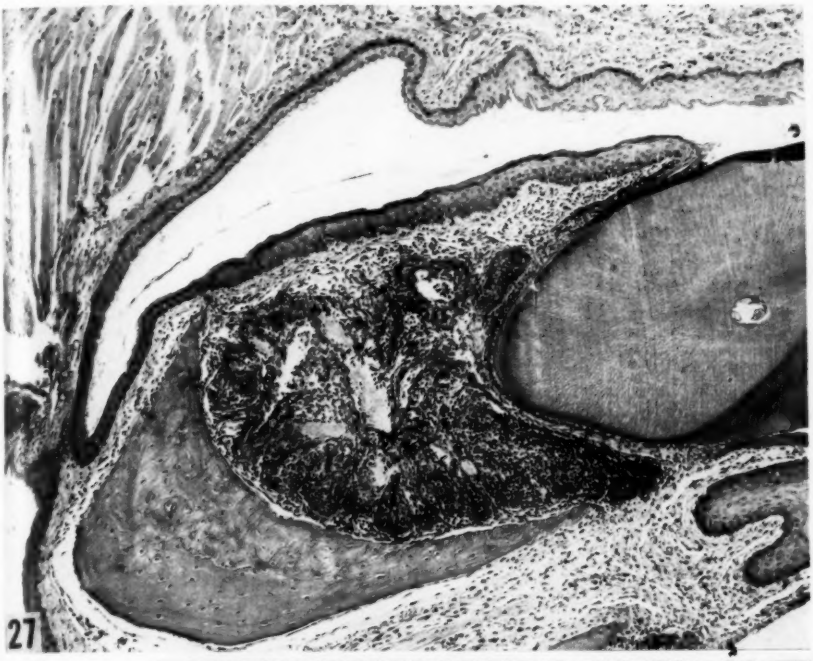
PLATE 63

FIGURE 27.—Tumor apparently originating from odontogenic epithelium, in 4.5-month-old C3Hf/Bi mouse that received at birth short-term culture material of leukemia (L-12289). Tumor is situated between maxillary bone and tooth root of upper incisors. Pattern resembles cystic basal-cell epithelioma. × 90

FIGURE 28.—Tumor resembling ameloblastoma in mandible of 2.5-month-old (C3Hf × AKR)F_1 mouse that received first passage of PTA in P388 D_1 culture. Ameloblast-like cells palisaded in *upper right* and at *left;* stellate reticulum at *middle right;* squamous epithelium in *upper center* and, with keratinization, at *far left.* × 430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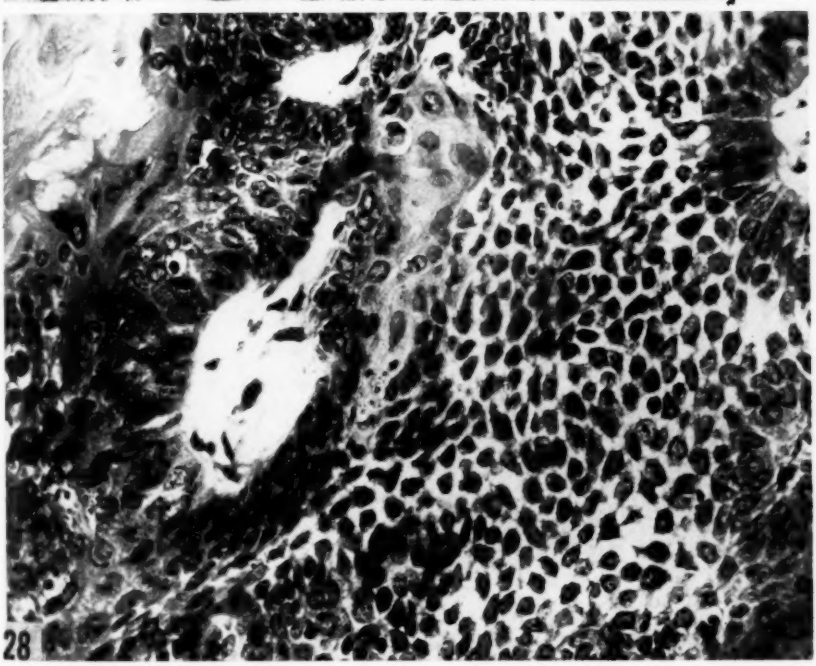
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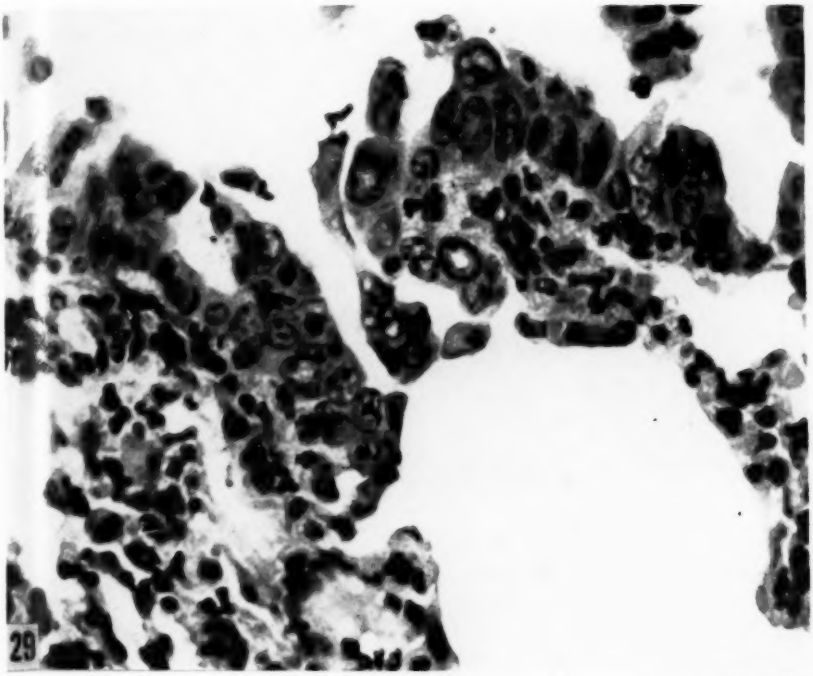
PLATE 64

FIGURE 29.—Lesion of terminal bronchiolar epithelium in 32-day-old AKR mouse that received first passage of PTA in P388 D_1 culture. *Note* disorganization of epithelium, large nuclei and nucleoli, and some nuclei with clear, vacuole-like areas. A few normal bronchial epithelial cells are present at *upper left*. Alveolar duct at *lower right*. × 690

FIGURE 30.—Lesion of terminal bronchiolar epithelium in 57-day-old C58 mouse that received same material as animal illustrated in figure 29. Epithelial changes are similar to those in figure 29. Normal bronchial epithelium in *left center*. × 430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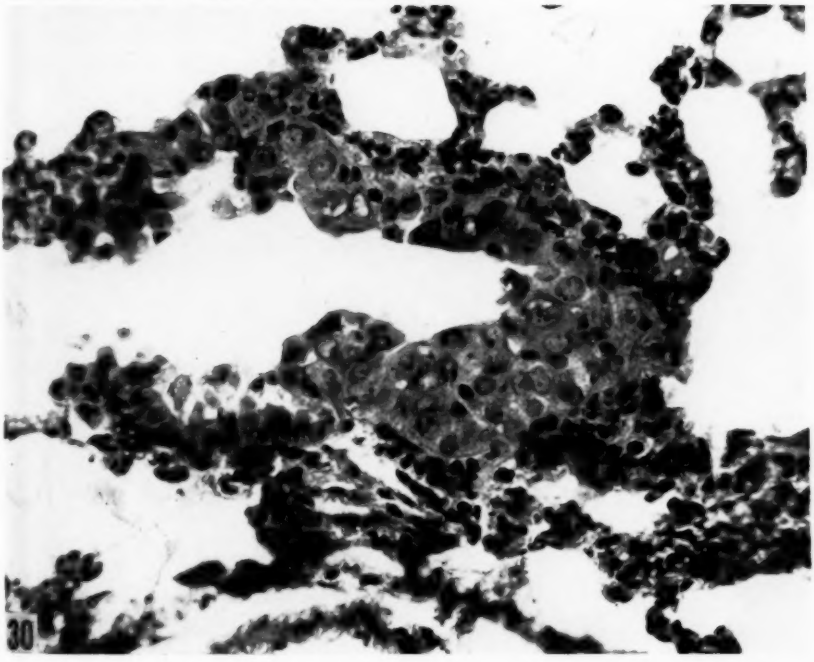
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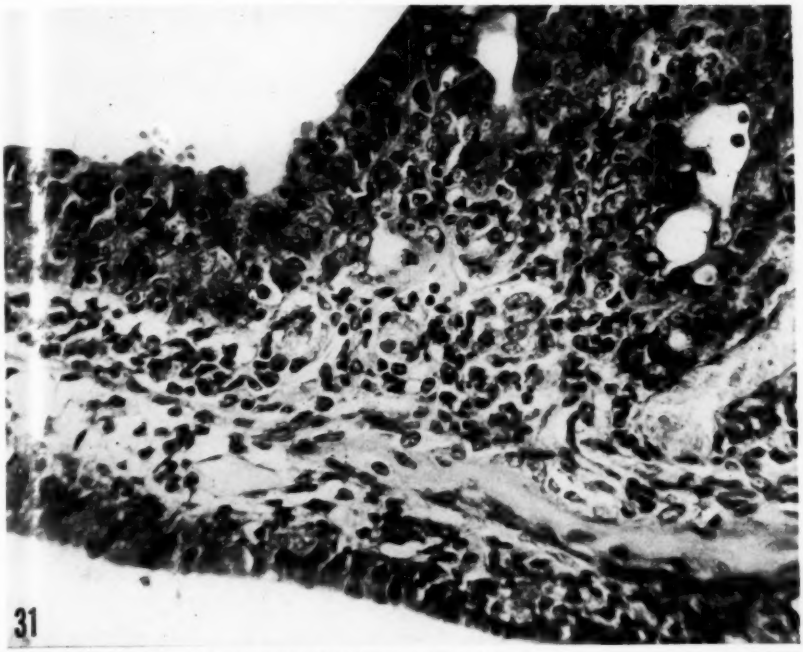
PLATE 65

FIGURE 31.—Proliferative lesion of surface epithelium of nasal mucosa in 2-month-old RFM mouse that received first passage of PTA through P388 D_1 culture. Disorganized epithelial cells with enlarged nuclei on the *upper* surface of turbinate process, normal epithelium on *lower* surface. × 430

FIGURE 32.—Intravascular pulmonary tumor embolus from a salivary-gland tumor in a 2.5-month-old C3Hf/Bi mouse that received short-term culture of thymic tumor (TT-427, table 9). Glandular pattern is evident in the tumor embolus, which is separated from the lower wall of the vessel. × 410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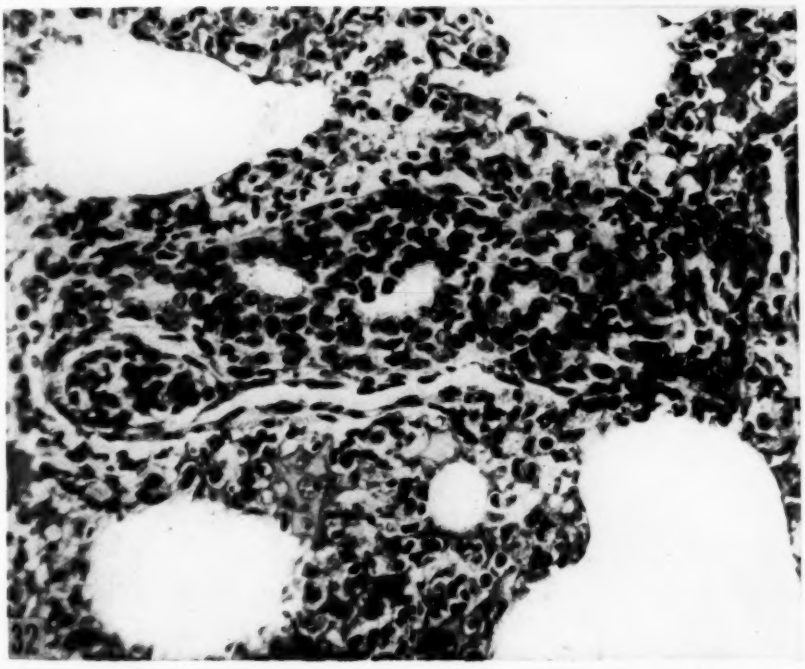
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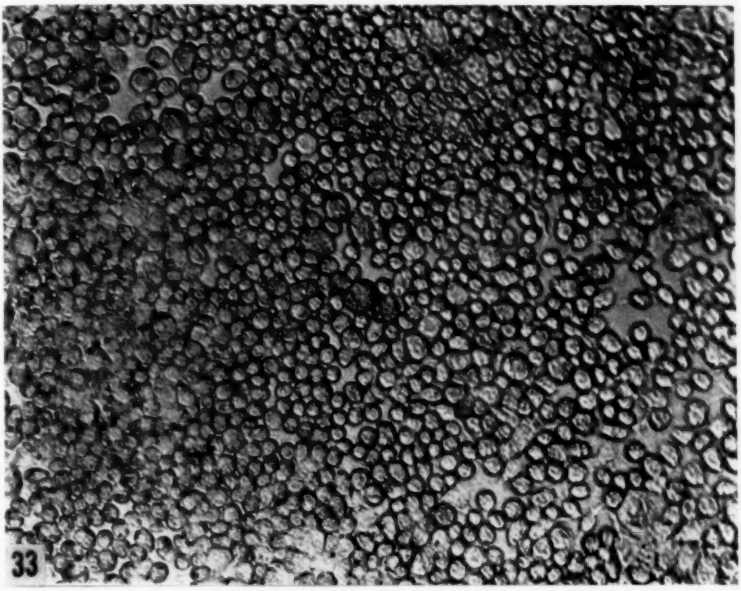
PLATE 66

FIGURE 33.—Living control culture of P388 D_1 continuous cell strain in medium LN40. × 200

FIGURE 34.—Living P388 D_1 culture showing cytopathogenic effect 6 days after inoculation with fifth passage of PTA in this cell line. *Note* clumping of cells and multinucleated giant cells. × 200

FIGURE 35.—Living P388 D_1 carrier culture 7 months after a single exposure to PTA. *Note* grouping of cells into spheres and rosettes, and extension of long filamentous cytoplasmic processes. × 200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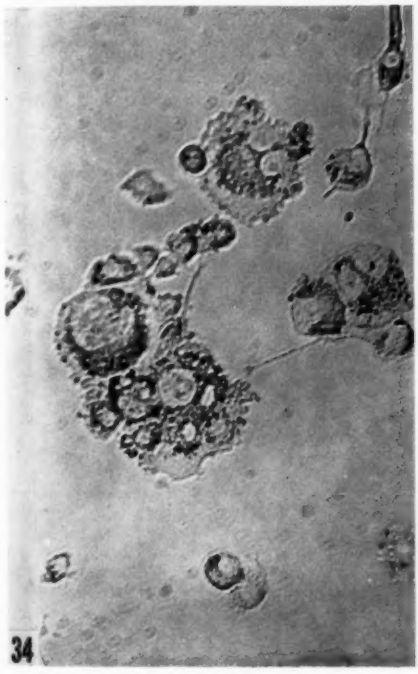
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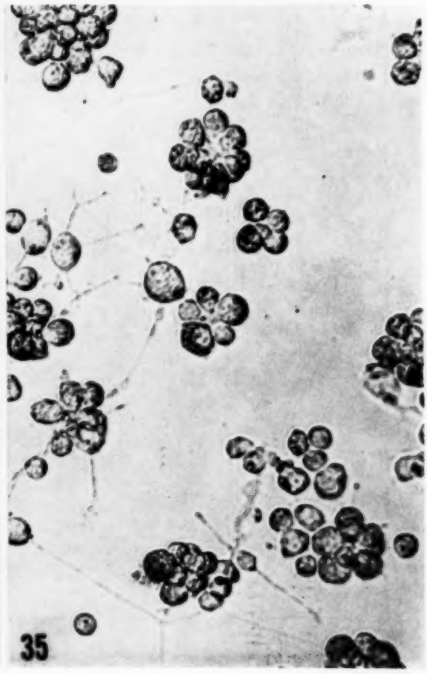
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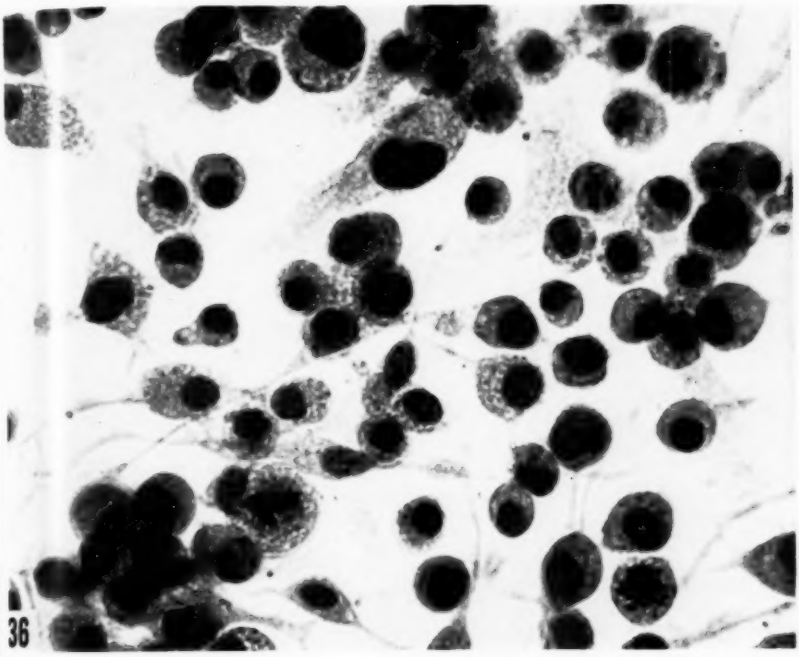
PLATE 67

FIGURE 36.—Fixed and stained coverslip culture of P388 D_1 control. Hematoxylin and eosin. × 690

FIGURE 37.—Fixed and stained coverslip culture of P388 D_1 cells showing cytopathogenic effect 6 days after inoculation with fifth passage of PTA in this cell line. Giant cell shows numerous focal areas in cytoplasm with increased intensity of staining by eosin. Adjacent cells show multiple cytoplasmic "buds." Hematoxylin and eosin. × 570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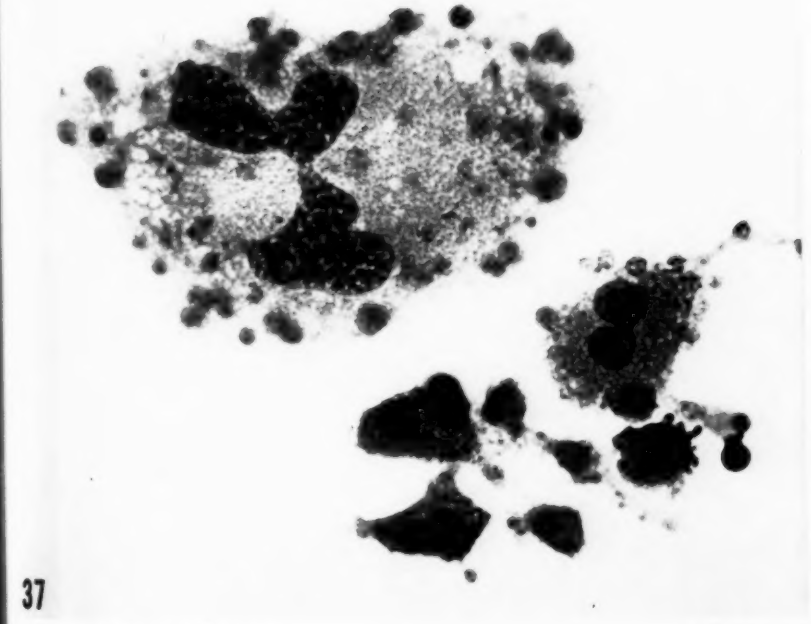
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Localization of Radioiodinated Rat Fibrinogen in Transplanted Rat Tumors [1, 2]

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SUMMARY

Radioiodinated rat fibrinogen localized in three different transplantable rat tumors during the initial inflammatory reaction of the host to the implant and during the rapid growth stage of the tumor. Extent of localization during inflammation paralleled the amount of tissue inflamed; extent of localization during subsequent tumor growth paralleled rate of tumor growth. In the Murphy-Sturm lymphosarcoma at its most vigorous state (10 gm., 8 days after implantation), 40 percent of the intravenously injected fibrinogen localized in the tumor in 6 hours as the tumor grew nearly two more grams. Disappearance of labeled fibrinogen from the tumors was slower than that of labeled fibrinogen from the blood, but when antifibrin antibodies were present, the disappearance of labeled fibrinogen from both was markedly hastened without a parallel disappearance of antibody.—J. Nat. Cancer Inst. 23: 799–812, 1959.

IN A previous report we showed that radioiodinated rat fibrinogen localized, presumably by conversion to and fixation as fibrin, in the Murphy-Sturm rat lymphosarcoma and in three other transplanted rat tumors (*1*); however, the focus of our attention in those studies had been on antibody directed against fibrinogen and fibrin, and on the mode of localization of such antibody in rat tumors. Spar, Bale, and Goodland (*2–4*) have confirmed those findings.

In the experiments described here, we examined in more detail the localization of radioiodinated fibrinogen itself at different stages in the growth of transplanted rat tumors, including the initial inflammatory reaction during the first few days after implantation and the terminal growth phase leading to death of the tumor-bearing host. We also measured the localization of fibrinogen in the inflamed areas of rats, in which implants of sterile gauze had been made to ascertain the extent of fibrinogen fixation during a typical, nonspecific, foreign-body reaction.

[1] Received for publication March 30, 1959.

[2] This work was supported in part by a contract with the U.S. Atomic Energy Commission, and was presented at a meeting of the American Association for Cancer Research, Atlantic City, April, 1959.

[3] The authors wish to acknowledge the assistance of Miss Marilyn Sanden, Miss Janice Orr, and Mr. Eugene Kanski.

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EXPERIMENTAL PROCEDURES

Preparation and properties of radioiodinated rat fibrinogen.—The details of preparation and radioiodination of the rat fibrinogen used here have been described (*1*). The concentration of each fibrinogen solution after iodination (*ca.* 20 mg. in 20 ml.) was brought to 0.1 mg. per ml. (tenfold dilution) with borate-buffered (*p*H 8) 0.9 percent NaCl. Dialyzable radioactivity was less than 0.5 percent in each case. Clottability of the fibrinogen preparations with thrombin ranged from 87.4 to 89.6 percent as based on radioactivity counts. The specific radioactivity of each preparation (approximating 10 μc./mg. fibrinogen) remained constant after precipitation from NaCl (3.16 M). The intravenous dose given each rat was 2.0 ml. containing 0.2 mg. and 2 μc. radioiodinated fibrinogen. No carrier protein was added.

The dilute radioiodinated fibrinogen solutions were stable *in vitro* at 8° C., with respect to clottability and recovery tests, for 2 weeks or more; however, there appeared to be some change even after only 4-day storage, with respect to an additional test carried out *in vivo* in normal rats. In terms of the intravenously injected radioactive dose, the average amount of radioactivity for freshly iodinated material still present in the blood of twenty-four 100-gm. rats, after 1 day, was 7.5 ± 0.9 percent ml.; the average amount in whole thyroid, 1.5 ± 1.1 percent. For preparations stored for more than 4 days before injection, the levels of radioactivity in blood assayed lower; in thyroid, higher. As an extreme case, only 1.8 percent per ml. of one 2-week-old preparation, when assayed *in vivo* in 4 rats, was found in the blood; in the thyroid, 13 percent. To insure uniformity throughout the experiments, preparations were not used if they were in solution more than 3 days after iodination, and each was tested routinely for clottability and dialyzability. Such caution was necessary here since, in tumor-bearing animals, the blood levels of radioactivity could not reliably serve as indexes of protein integrity. In such animals, at certain stages of tumor growth, blood levels of radioactivity for even freshly iodinated fibrinogen may be as low as 75 percent of normal.

When plasma was obtained from several normal rats one-half hour after injection of radioiodinated fibrinogen and was clotted, the percentage of plasma radioactivity in the clot was the same as the clottability index (88.5%) of an uninjected portion of the labeled preparation which had been diluted with rat plasma to simulate conditions *in vivo*. Plasma obtained from the same normal rats 1 day after injection had a lower specific activity (75% of the ½-hour value) but a clotting index practically unchanged (90.2%). This showed that the iodinated fibrinogen did not contain degraded labeled components, which remained behind in the circulation as the fibrinogen was slowly catabolized, and that labeled fragments, after catabolism, did not remain in the circulation for very long.

Assay of radioiodinated fibrinogen in tumor-bearing rats.—Rats were injected intravenously (in the tail vein) with 2.0 ml. portions, 0.2 mg., of

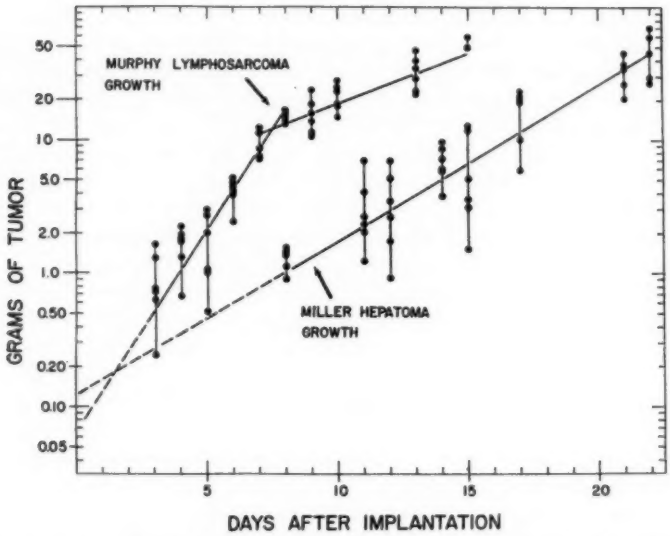
radioiodinated fibrinogen containing about 2 μc of I^{131}. They were killed 17 to 20 hours later (unless otherwise indicated) under Nembutal anesthesia (heparin added) by retrograde perfusion into the aorta with 0.9 percent NaCl. Venous blood, sampled just prior to perfusion, and perfused tissues were assayed for radioactivity in a well-type scintillation counter. The tumor was divided into 0.5- to 1.0-gm. portions and all parts were counted, since the nonuniform distribution of radioactivity in the tumor made adequate sampling impossible. The other tissues were either small enough to be counted *in toto* or, as in the liver, uniform enough with respect to distribution of radioactivity to permit reliable sampling. The radioactivity in the blood was determined by counting 1-ml. portions of whole blood and multiplying by the circulating blood volume, taken as 6 percent of the rat weight for short-term experiments (less than 5 hours) or 10 percent for longer times of circulation to account for the equilibration of the whole protein space.

Tumor-bearing rats.—Male Wistar (Carworth) rats, about 120 gm. in weight, were used as hosts for the Murphy-Sturm lymphosarcoma; male Sprague-Dawley (Holtzman) rats, also about 120 gm. in weight, were used as hosts for the Miller and Simpson tumors and for other miscellaneous inoculums.

Rats were inoculated subcutaneously with tumors by trocar in the right axillary region. The volume of implanted tissue was routinely kept within the limits of 0.05 to 0.15 ml. For the experiments in which a large number of rats in several groups were needed and in which the members of the groups were to be assayed at different times after implantation, all rats were inoculated under aseptic conditions within the shortest time possible, with the same well-established, healthy, nonnecrotic donor tumor. For example, if 72 tumor-bearing rats were required, 72 trocars would first be loaded and then within a 5-minute period all the rats would be inoculated. Immediately after inoculation the rats would be divided into groups for the remainder of the experiment. Such a procedure prevented selection of particular animals for any particular group.

The origin and maintenance of the solid lines of tumors have been described previously (*5*, *6*). They were the fast-growing Murphy-Sturm lymphosarcoma, which we had utilized more than any other in our antibody studies, the Miller hepatoma for which we had both fast- and slow-growing lines, and the Simpson reticulum-cell sarcoma for which we also had two lines, one fast- and the other slow-growing.

Tumor growth rate was measured by daily caliper measurements of two diameters of the palpable masses, and, at the time of assay, direct weighing of the excised tumor mass. Since in many instances weighings immediately followed palpations and since these both were done at various stages of tumor development, it was possible to relate palpation data (product of two diameters) to tumor weight, and, hence, to measure the day-to-day progress of every tumor in terms of its weight. Rates of tumor growth were obtained from the palpation data (shown in text-fig. 1 as least-square lines after conversion from palpation to weight units).

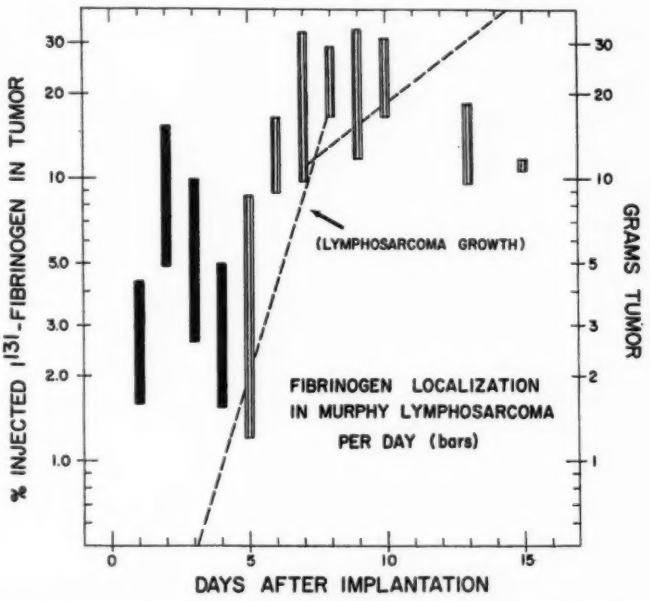


TEXT-FIGURE 1.—Growth of the Murphy-Sturm lymphosarcoma and the Miller hepatoma. Lines are plots of least-square equations derived from daily caliper measurements of tumors; groups of points show weights of tumors at time of assay for experiments described in text.

Actual weight data obtained for the various groups at the time the rats were killed are also shown in text-figure 1. Growth data, taken within a few days after tumor implantation, were not included in the figure since they were not true measurements of tumor growth but rather of an inflammatory response, as shown in experiments later.

EXPERIMENTAL RESULTS

Localization of fibrinogen in the Murphy-Sturm lymphosarcoma.—Seventy-two Wistar rats were inoculated with the Murphy tumor and divided by random selection into 12 groups of 6 each. The data for the Murphy tumor (text-fig. 1) show the weights of each tumor of each group at the time of assay except for the first 2 groups which contained little tumor but much solid inflamed tissue. The animals were injected with I^{131}-fibrinogen the day before assay. The bars in text-figure 2 show the range of localization values obtained for fibrinogen in each group. Two phases of localization can be noted, one (black bars) with a peak (as high as 15.7%) 2 days after implantation of the tumor, and the other with a peak (as high as 33.4%) some 7 days after. The first phase can be interpreted as an inflammatory reaction to a foreign body and the second as fibrinogen fixation during growth of the tumor itself. Finally, as the

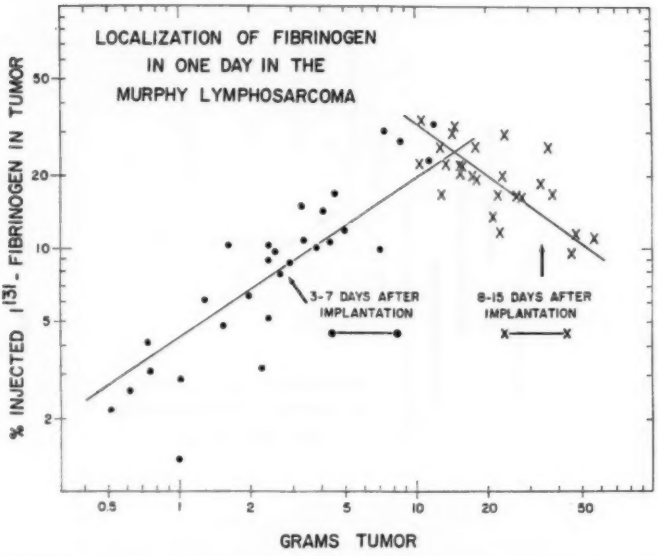


TEXT-FIGURE 2.—Fibrinogen localization in the Murphy-Sturm lymphosarcoma. I^{131}-rat fibrinogen was injected in each instance 1 day before assay. *Black bars* show inflammatory reaction; *striped bars*, localization in tumor itself; *dotted line* shows growth of tumor.

tumor growth rate decreases, daily fibrinogen fixation becomes less. The tumor size rather than time after implantation appears to have the greater physiological control over extent of localization as shown in text-figure 3 where the percentage of fibrinogen fixed is plotted as a function of tumor size rather than age. In reality, it is the amount the tumor grows during the period between injection and assay which determines the amount localized.

Several phenomena take place at the time the juncture in text-figures 2 and 3 occurs, that is, at the time fibrinogen localization reaches a peak and begins to decline: The tumor growth rate decreases, blood levels of radioactivity start rising, host weight (total weight minus tumor weight) ceases to increase and begins to decrease slowly but steadily, and central necrosis of the tumors ensues. The host enters a period of steady decline and dies sometime between the beginning of the 12th and the end of the 15th day after implantation. These data are summarized in table 1.

Localization of fibrinogen in the Miller hepatoma.—Seventy-two Sprague-Dawley rats were inoculated with the Miller tumor and divided into groups as previously described. The sizes of the tumors of each group at the various times of assay are shown in text-figure 1; the localizations of fibrinogen (1 day between injection and assay) in these tumors at the



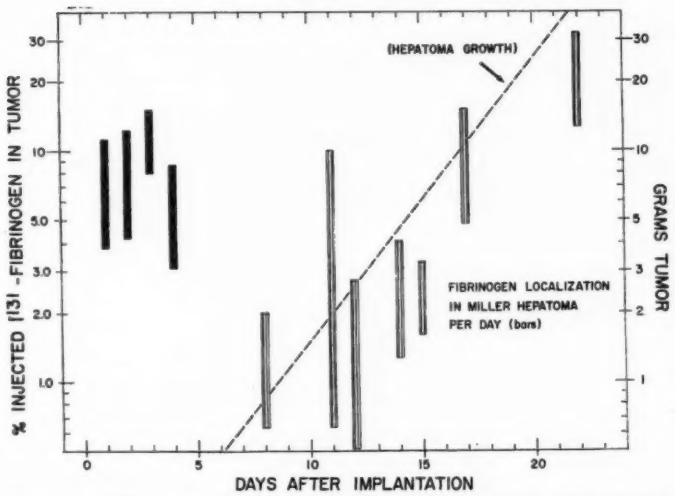
TEXT-FIGURE 3.—Fibrinogen localization in the Murphy-Sturm lymphosarcoma as a function of tumor size. Data obtained during inflammatory reaction are not included. I^{131}-rat fibrinogen was injected in each instance 1 day before assay.

TABLE 1.—Localization of I^{131}-rat fibrinogen in rats bearing Murphy and Miller tumors (values averaged from 6 rats, 17 to 20 hours after injection)*

Tumor	Days after implantation	Host weight (gm.)	Tumor weight (gm.)	Percentage injected dose† in: Tumor	Liver	Blood‡
Murphy-Sturm lymphosarcoma	1	119	(0.1) §	1.9	0.40	62
	6	130	4.1	11.3	0.40	45
	7	133	9.3	24.7	0.38	19
	8	129	14.7	23.2	0.38	20
	15	100	53.1	11.1	0.42	52
Miller hepatoma	1	124	(0.1) §	7.8	0.43	58
	3	131	(0.3) §	12.1	0.42	47
	8	168	1.3	1.2	0.47	61
	15	192	6.7	2.3	0.47	59
	17	193	10.9	8.3	0.42	47
	22	196	44.8	22.4	0.48	28

*See text-figures 1 to 6 for additional information.
†Values for kidney and spleen were all 0.1 percent; for thyroid, 0.5 to 2.5 percent.
‡Blood volume taken as 10 percent of (host plus tumor) weight.
§True tumor weight estimated from text-figure 1 to exclude weight of inflamed tissue.

various times after implantation are shown in text-figure 4; the localizations, plotted as a function of tumor weight, are shown in text-figure 5. Again two phases of localization can be noted: an inflammatory reaction (black bars) during the first few days after implantation, this time with

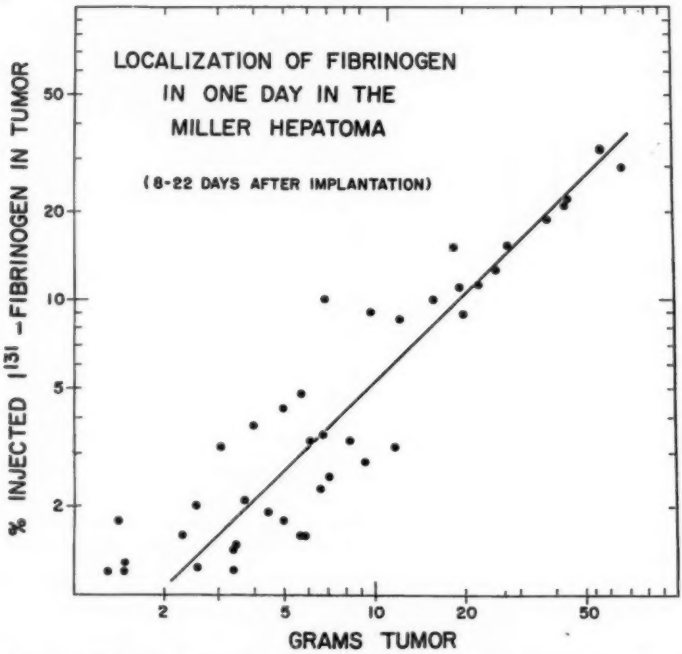


TEXT-FIGURE 4.—Fibrinogen localization in the Miller hepatoma. I^{131}-rat fibrinogen was injected in each instance 1 day before assay. *Black bars* show inflammatory reaction; *striped bars*, localization in tumor itself; *dotted line* shows growth of tumor.

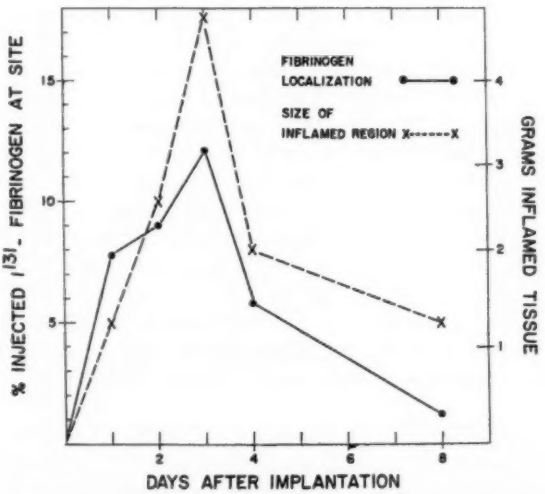
a peak response (as high as 15.4%) on the 3d day; and a second phase several days later which builds up to a maximum response (as high as 32.8%) on the 22d day. In contrast to the Murphy-Sturm lymphosarcoma, the 50-gm. tumors localized the largest amount of fibrinogen and appeared the most vigorous at this juncture. At this point also the host weight (total minus tumor weight) ceased to increase. Immediate death rather than general decline ensued. These data are summarized also in table 1. The inflammatory response immediately following implantation is shown in more detail in text-figure 6 in which the amount of inflamed tissue at various times after inoculation of the tumor is shown. A direct relation between the fibrinogen localization and the size of the inflamed region can be seen. The amount of tumor present can be estimated from the extrapolated growth curve of text-figure 1, *e.g.*, 0.3 gm. on the 3d day, surrounded by 5 gm. of well-vascularized inflamed normal tissue.

Localization of fibrinogen in the Simpson reticulum-cell sarcoma.—Slow- and rapid-growing lines of the Simpson reticulum-cell sarcoma each gave rise to inflammatory responses soon after injection (table 2). Tumors of the slow-growing variety, however, which were of the same size (20-gm. region) as the fast-growing type, localized much less fibrinogen (7% *vs.* 18%) as shown in table 2. This emphasizes that it is the amount of growth between injection and assay which determines the amount localized.

Localization of fibrinogen in inoculums of sterile gauze and of normal liver.—Subcutaneous areas, in which sterile gauze (12 thicknesses, 5 × 40 mm.) and rat liver fragments were implanted, contained fixed fibrinogen, with a peak response 1 day after inoculation (table 3). The liver, however, which was rapidly absorbed and which, in any event, did not en-



TEXT-FIGURE 5.—Fibrinogen localization in the Miller hepatoma as a function of tumor size. Data obtained during the inflammatory reaction are not included. I^{131}-rat fibrinogen was injected in each instance 1 day before assay.



TEXT-FIGURE 6.—Fibrinogen localization during inflammatory reaction to the Miller hepatoma. Points are each averaged from values for 6 rats.

TABLE 2.—Localization of I^{131}-rat fibrinogen in rats bearing slowly and rapidly growing lines of the Simpson reticulum-cell sarcoma (values averaged from 6 rats, 17 to 20 hours after injection)

Tumor line	Days after implantation	Host weight†	Tumor weight	Percentage injected radioactive dose found in:* Tumor site	Normal fascia‡	Liver	Blood§
One (slow growing)	1	124	—	9. 1	0. 2	0. 4	63
	2	140	—	2. 9	0. 2	0. 5	65
	3	141	—	1. 4	0. 1	0. 4	70
	4	148	—	1. 1	0. 1	0. 4	74
	52	289	21. 0	7. 0	—	0. 5	69
Two (fast growing)	2	129	—	4. 2	0. 2	0. 5‖	71
	11	157	18. 7	18. 6	—	0. 4	24

*Spleen and kidney localizations were all 0.1 percent of the injected dose; thyroid average 1.8 percent.
†Total weight minus tumor weight.
‡Taken from opposite side of rat (approximates size of inflamed tissue).
§Blood volume estimated as 10 percent (host plus tumor) of weight.
‖Value for 3 rats; the other 3 had liver metastases (*see* text).

TABLE 3.—Inflammatory response to implantation of sterile gauze or normal rat liver as measured by localization of I^{131}-rat fibrinogen (values averaged from 6 rats, 17 to 20 hours after injection)

Inoculum	Days after implantation	Host weight	Percentage injected radioactive dose found in: Normal fascia*	Liver	Blood†	Site of implant
Sterile gauze	1	122	0. 2	0. 5	48	19. 9
	2	128	0. 4	0. 4	53	11. 3
	3	130	0. 2	0. 5	61	5. 1
	4	137	0. 2	0. 3	64	5. 2
Normal liver	1	121	0. 1	0. 4	57	3. 0
	2	125	0. 1	0. 5	65	2. 7

*Taken from opposite side of rat (approximates size of inflamed tissue).
†Blood volume estimated as 10 percent (host plus tumor) of weight.

gender vascularization, produced only a low and short-lived response. The gauze caused the highest fibrinogen fixation (as high as 24.2%) of all inflamed areas measured including the tumor.

Localization of fibrinogen in liver metastases of the Murphy-Sturm lymphosarcoma and of the Simpson reticulum-cell sarcoma.—In 3 different rats from 3 different isolated experiments in which I^{131}-fibrinogen was assayed in rats bearing the Murphy tumor, liver metastases (later proved by histologic examination) were encountered (11, 12, and 13 days after subcutaneous implantation). In each instance, after perfusion, 18 to 22 percent of the injected radioactive dose was found in the liver rather than the usual 0.4 percent. Other organs were tumor-free and did not localize fibrinogen above the usual low amounts.

Of the 6 rats, in table 2, which bore the slow-growing variety of the Simpson tumor, 3 had metastatic involvement (52 days after subcutaneous implantation) of the liver and lung and could be only partially perfused.

Fibrinogen localizations in these tumor-containing organs ranged from 0.6 to 2.6 percent and as such approached the values expected in normal but unperfused organs.

Of all normal rats (87) in which fibrinogen had been assayed, only 1 localized fibrinogen in its tissues. The liver of this animal, later diagnosed as having hepatitis, localized 22 percent of the injected fibrinogen.

I^{131}-fibrinogen content of the Murphy-Sturm lymphosarcoma as a function of time.—From the experiments described for the Murphy tumor, it can be seen that as the tumor grows, it continuously removes labeled fibrinogen from the circulation, and, at least for a short time, keeps it fixed within the tumor. This experiment was designed to determine the fate of the labeled protein as a function of time after its intravenous injection into rats bearing the Murphy tumor and, in particular, to determine the rate at which it becomes fixed and subsequently catabolized.

In the first part of the experiment (part A of table 4), fibrinogen was injected 1 day after tumor inoculation into 36 rats divided into 6 groups. The data show that the amount of I^{131}-fibrinogen in the tumors remained fairly constant as the tumors grew from 0.1 to 10 gm. and as the radioactivity in the blood slowly diminished during that 7-day period. From text-figure 2, we know roughly how much labeled fibrinogen was localized from the circulation each day as the tumor grew, and, hence, can integrate for the total amount of I^{131}-fibrinogen which should have localized in the tumor during the week. This calculated value is 20 percent of the injected radioactive dose. The observed value of 5 percent indicates that during the period under study tumor radioactivity remained constant, probably in a steady state between fixation of circulating radioactivity and catabolism (and rapid elimination) of the fixed material.

In the second part of the experiment (part B of table 4), fibrinogen was injected 7 days after tumor inoculation into 36 more rats divided into 6 groups. The data show that the large amount of labeled fibrinogen which was localized in a day's time at the peak of tumor vitality did not persist but gradually diminished during the days that followed as the tumor continued to grow from 10 to 50 gm.

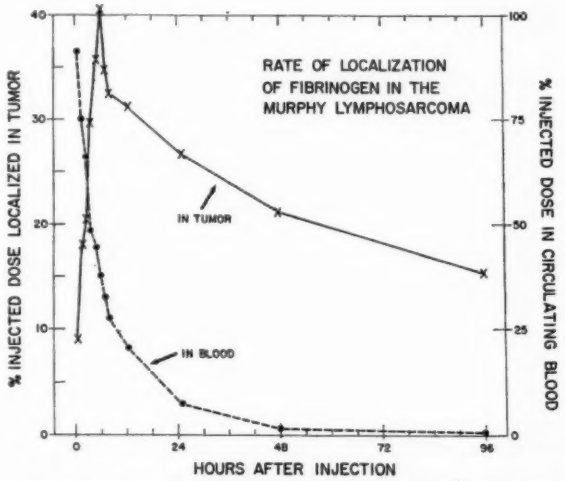
In the third part (text-fig. 7) of the experiment, fibrinogen was injected 7 days after tumor inoculation into 46 rats, which had been selected from a group of 80 tumor-bearers on the basis both of a previous uniform tumor growth rate and for a uniform size (10.0 gm.) at the beginning of the experiment. Individual rats were assayed during the first 8 hours in ¼-hour intervals (but taken as hourly groups of 4), and then groups of 4 or more rats were assayed at 12½, 25, 48, and 96 hours. The data of text-figure 7 show that the rate of localization of radioiodinated fibrinogen at the peak of tumor vitality was fairly rapid, 7 percent per hour. Catabolism of the newly localized material was noticed about 6 hours after injection and thus appears to have limited the maximum of localizability to about 40 percent of the injected radioactive material (43, 43, 39, and 38% localized in the 6th hour in 4 rats). The amount of radioactivity in the circulation was dropping rapidly as localization took place and

TABLE 4.—Localization in and disappearance from the Murphy lymphosarcoma of radioiodinated rat fibrinogen

Experiment	Days after tumor implantation	Weights (gm.)		Localization of fibrinogen* (percentage of injected radioactive dose):			
		Host†	Tumor	Tumor	Liver	Thyroid	Blood‡
Part A (rats injected with fibrinogen 1 day after tumor implantation)	2	115	(0.50)§	6.0	0.4	0.5	69
	3	115	(1.27)	5.1	0.2	2.2	35
	4	124	(2.51)	4.0	0.1	4.1	22
	5	133	(3.15)	5.7	0.1	3.7	18
	6	135	4.0	4.1	0.1	2.7	10
	7	135	10.1	6.3	0.0	5.3	5
Part B (rats injected with fibrinogen 7 days after tumor implantation)	8	119	10.4	32.7	0.4	0.4	21
	9	128	15.6	26.4	0.2	1.9	15
	10	136	19.2	19.2	0.1	2.4	9
	11	134	24.5	16.1	0.1	3.5	4
	12	130	28.7	10.9	0.1	4.1	3
	15	111	48.9	4.7	0.0	2.9	1

*Spleen and kidney values all were one quarter of the liver values.
†Total weight minus tumor weight.
‡On basis of blood volume of 10 percent (host plus tumor) of weight.
§Weights in parentheses are for tumor plus inflamed normal tissue.

reached a level of 8 percent in the circulation (circulating blood volume taken as 6 percent of the rat weight) in 24 hours (text-fig. 7). From palpation data taken throughout the 1st day of the experiment (which were converted to units of weight by the method described above), it was found that the tumors at this particular time were growing at an average rate of about 0.30 gm. per hour.



TEXT-FIGURE 7.—Fibrinogen localization in the Murphy-Sturm lymphosarcoma and disappearance therefrom and from blood as a function of time. Points are each averaged from values for 4 or more rats. Volume of circulating blood was found to be 6.06 percent of rat weight based on extrapolated zero-time value of radiolabeled fibrinogen.

Effect of anti-fibrin antibody on the localization of fibrinogen in the Murphy lymphosarcoma.—We had observed previously (*1*) that when I^{131}-labeled anti-rat-fibrin antibodies and I^{133}-labeled rat fibrinogen were injected together in tumor-bearing rats, the disappearance of the labeled fibrinogen from the tumor and the circulation was faster than that of the antibody. To check whether antibody affects the rate of I^{133}-fibrinogen clearance, several different I^{131}-labeled globulin preparations, some with antibody and others without, were paired with I^{133}-labeled rat fibrinogen, and each pair was assayed in a group of tumor-bearing rats in accordance with previously established protocols (*1*). The results, in table 5, show quite conclusively that antibody does indeed markedly increase the rate of fibrinogen removal without being removed itself. Normal globulin is ineffective.

TABLE 5.—Localization of radioiodinated protein pairs in rats bearing the Murphy lymphosarcoma in which one member of pair is rat fibrinogen (rats assayed 19 hours after injection of pairs)

Number rats assayed	Average tumor weight (*gm.*)	Protein pairs (I^{131}-*labeled protein above* I^{133}-*labeled protein*)	Localization of protein pairs in the rat* (percentage of injected radioisotopic dose):			
			Tumor	Liver	Thyroid	Blood†
6	9.8	Purified anti-rat fibrin, rabbit	29	1.8	0.7	32
		Rat fibrinogen	9	0.4	4.0	13
6	11.9	Unpurified anti-rat fibrin, rabbit	9	1.3	0.7	28
		Rat fibrinogen	8	0.3	2.4	11
24	8.8	Normal γ-globulin, rabbit	6	1.2	1.0	21
		Rat fibrinogen	25	0.3	1.2	23
8	9.9	Normal γ-globulin, pig	8	0.1	0.4	29
		Rat fibrinogen	27	0.3	0.9	19
12	15.0	Normal γ-globulin, rat	8	0.4	0.8	29
		Rat fibrinogen	23	0.4	2.4	21

*Calculated as shown in (*7*).

†Based on estimated blood volume of 10 percent of rat weight.

DISCUSSION

The experiments described here show the role played by fibrinogen during the growth of certain transplanted tumors in rats. First there is an inflammatory response during which fibrinogen is rapidly laid down, presumably an integral part of the host's defense against foreign bodies. This is shown here to manifest itself not only in a reaction to tumor but also to a foreign body such as sterile gauze. In transplantable tumors, the fibrinogen fixed as fibrin may serve not only as a host defense but also paradoxically to protect the tumor from the host; *i.e.*, it may serve to keep the tumor separated from the host (and the host defenses) and to provide support and nutrient during the early growth stages. In any event, that tumor growth was steady, in spite of the inflammatory response, is indicated by extrapolation of the growth curves for the Murphy and Miller tumors (text-fig. 1) back to the time of inoculation. A time

zero intersection at the ordinate shows an amount of tumor matching a typical trocar implant (0.05–0.15 ml.). By the time inflammation subsides the tumor has enough autonomy to survive under host attack and to continue to grow.

As tumor growth continues unabated, fibrin is also laid down in increasing amounts, as shown by the amount of I^{131}-fibrinogen localized. That this fibrinogen is fixed as fibrin and is not merely trapped as serum protein is shown by the fact that labeled globulin is present to only the maximum extent of 7 percent of the injected dose per 10 gm. of tumor (*5*), while labeled fibrin at the same stage is present to as much as 40 percent. The 7 percent may be taken as a maximum figure for the fraction of unfixed plasma proteins in a 10-gm. tumor.

Continued growth of tumor causes the host to enter a period of negative nitrogen balance and cachexia (*8*), while the tumor, now unable to get enough nutrition, no longer is able to continue growing at the same rate, and this is reflected in the decreased fibrinogen uptake in such tumors as found here.

It is interesting to calculate the nutrient requirement for growth of the Murphy-Sturm lymphosarcoma at its stage of maximum growth rate, *i.e.*, 0.3 gm. per bour. It is obvious that about 60 mg. (dry wt.) of protein or equivalent is required per hour to maintain such growth. If this comes from the circulation (plasma protein, 60 mg./ml.), 1 ml. of plasma would be required per hour and represents about 15 percent of the whole plasma pool. Some of the protein may be used directly by the tumor, but since we find only about 7 percent of a dose of I^{131}-globulin in the tumor at any one time, it is obvious that the tumor does not incorporate very much of the iodine label. This further emphasizes that the high localization of I^{131}-fibrinogen is due to actual deposition of I^{131}-fibrin and not to reincorporation of the iodine label.

Fibrin itself or at least the iodine label is removed from the tumor slowly after deposition, dropping to one half its value in about 4 days. Iodinated fibrinogen is cleared from the blood of the rats bearing the rapidly growing tumors very much more rapidly, with a half-life of about 5 hours.[4] All this turnover in the blood is not due entirely to fixation in the tumor but partially due to rapid metabolism. The rate of degradation of the iodinated fibrin was found to be much greater in the presence of anti-fibrin antibody.

It is instructive to compare the dynamic picture of tumor growth which Algire (*9*) has given us (by the Chalkley technique (*10*)) with the data shown here. In that picture, there was a latent period of 3 days before new capillary sprouting occurred in a C3H mouse mammary tumor, which corresponds to the inflammatory response period here. Then, 4 days before the tumor growth rate slowed down, a stable vascular development was attained. On the 12th day after implantation, Algire found that the tumor became surrounded by a leukocytic ring and that from that point on growth continued at a slower but constant rate. This

[4] This rate is much greater than that in non-tumor-bearing animals (*see* p. 800).

4-day period before change in growth rate corresponds well with the active laying down of fibrin in the Murphy and Miller tumors in which, *per gram*, the fibrin localization is constant during the days leading up to the peak of tumor vitality and the peak of fibrin localization. If the host does not die (as it does with the Miller hepatoma), the tumor now begins to suffer from its dependence on the host—its growth slows down, it becomes necrotic in parts, and it presents a toxicity problem for itself and the host.

REFERENCES

(*1*) DAY, E. D., PLANINSEK, J. A., and PRESSMAN, D.: Localization *in vivo* of radioiodinated anti-rat-fibrin antibodies and radioiodinated rat fibrinogen in the Murphy rat lymphosarcoma and in other transplantable rat tumors. J. Nat. Cancer Inst. 22: 413–431, 1959.

(*2*) SPAR, I. L., BALE, W. F., and GOODLAND, R. L.: *In vivo* localization studies of I^{131} labeled anti-Murphy-Sturm lymphosarcoma antibodies. Univ. Rochester Atomic Energy report UR–535: 1–23, 1958.

(*3*) SPAR, I. L., GOODLAND, R. L., and BALE, W. F.: Localization of I^{131} labeled antibody to rat fibrin in a transplantable rat lymphosarcoma. Univ. Rochester Atomic Energy report UR-536: 1–11, 1958.

(*4*) ———: Localization of I^{131} labeled antibody to rat fibrin in a transplantable rat lymphosarcoma. Proc. Soc. Exper. Biol. & Med. 100: 259–262, 1959.

(*5*) DAY, E. D., PLANINSEK, J., KORNGOLD, L., and PRESSMAN, D.: Tumor-localizing antibodies purified from antisera against Murphy rat lymphosarcoma. J. Nat. Cancer Inst. 17: 517–532, 1956.

(*6*) PLANINSEK, J. A., KUBAI, L., and DAY, E. D.: Five ascites rat tumors: their origin, conversion, and maintenance. Transpl. Bull. 6: 116–118, 1959.

(*7*) PRESSMAN, D., DAY, E. D., and BLAU, M.: The use of paired labeling in the determination of tumor-localizing antibodies. Cancer Res. 17: 845–850, 1957.

(*8*) TANNENBAUM, A., and SILVERSTONE, H.: Nutrition in relation to cancer. Advances Cancer Res. 1: 451–501, 1953.

(*9*) ALGIRE, G. H.: The transparent chamber technique as a tool in experimental tumor therapy. *In* Approaches to Tumor Chemotherapy (Moulton, F.R., ed.). Washington, D.C., Am. Assoc. Adv. Sc., 1947, pp. 13–25.

(*10*) CHALKLEY, H. W.: Method for the quantitative morphologic analysis of tissue. J. Nat. Cancer Inst. 4: 47–53, 1943.

Comparison of the Virulence of the Mammary-Tumor Agent From Four Strains of Mice [1, 2]

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SUMMARY

Mice of strain BALB/c, taken from their mothers within 24 hours after birth, were suckled by foster mothers of high-tumor strains C3H, A, DBA/1, and RIII. Controls were BALB/c mice nursed by their natural mothers. The 4 groups of foster-nursed mice, C_fC3H, C_fA, C_fDBA, and C_fRIII, differed only in the source of the mammary-tumor agent ingested with the milk; the unfostered mice were presumably agent-free. All mice were force-bred, and examined weekly for mammary tumors; all tumors were diagnosed and typed histologically. Incidences of mammary tumors were high in all fostered groups: 90.9 percent in 55 C_fC3H, 88.5 percent in 61 C_fA, 88.2 percent in 51 C_fDBA, and 79.2 percent in 53 C_fRIII; incidence was low (5.1%) in 78 control mice. Although incidences among the fostered groups were not significantly different, average tumor ages were. The average tumor age of the C_fC3H group was 7.8 months; the mean ages in the other three groups were 9.3 months in C_fA, 9.6 months in C_fDBA, and 10.7 months in C_fRIII. The agent in the milk of strain C3H mice induced tumors more rapidly in strain BALB/c mice than the agent in the milk of strains A, DBA/1, and RIII. There were indications, but not clear-cut evidence, that the agent in the milk of strain RIII mothers was less potent than that in strains A and DBA/1. These data are evidence that the mammary-tumor agent exists in forms which differ in their virulence or tumor-inducing capacities.—J. Nat. Cancer Inst. 23: 813–821, 1959.

HIGH INCIDENCES of mammary tumors in mice of certain inbred strains are known to be associated with an agent in the milk transferred from generation to generation (*1*). This agent, with the characteristics of a virus (*2*), induces mammary tumors in over one half of all female mice with genetic susceptibility to its action and with adequate hormonal stimulation. Mammary tumors developing in the absence of the agent are less frequent and tend to develop at a much later age (*3*).

Each high-tumor strain of mice has a characteristic incidence of mammary tumors and an average age at which females develop tumors. There are also colony and substrain variations in tumor incidences and ages, which have been attributed to environmental conditions as well as

[1] Received for publication April 2, 1959.
[2] Aided by grants from the American Cancer Society, Inc., and by a gift from Mrs. Edward Wood.

to differences in genetic constitution (*4*). In addition, there are evidences of agent variants differing in their ability to stimulate mammary-tumor development (*5*, *6*).

To test whether variants of the agent existed in our colony, mice of strain BALB/c, which have a low incidence of mammary tumors but a high susceptibility to action of the agent (*7*), were foster-nursed by mothers of 4 high-tumor strains: C3H, A, DBA/1, and RIII. Differences in mean age of tumor development were found among the 4 groups of foster-nursed mice, though all were of identical genotype, unrelated to foster mothers, and subjected to the same experimental and environmental conditions. The agent from strain C3H mothers induced tumors at an earlier age than the agent from the other high-tumor strains, and there were indications that the agent in strain RIII mice was somewhat less virulent than that in the others.

MATERIALS AND METHODS

Mice used in the experiment were of the following substrains: BALB/cScHu, C3H/HeHu, A/HeHu, DBA/1Hu, and RIII/JaxHu (*8*). BALB/c mice, also designated by the symbol C, were taken from their mothers within 24 hours of birth and given to foster mothers of the high-tumor strains. These BALB/c mice replaced the young that had been born at approximately the same time. The 4 fostered groups will be designated by the symbols C_tC3H, C_tA, C_tDBA, and C_tRIII. Controls were BALB/c mice nursed by their natural mothers. All mice in the colony were kept in one room, and received the same food and care. All mice of strain BALB/c used in the experiment were born between November and July; the choice of foster mothers was dependent only on their being tumor-free and having recently borne young.

At 3 to 4 weeks of age, the fostered animals were weaned and placed 4 in a pen with a BALB/c male (not fostered). Force-breeding, a method described by Bagg (*9*) and used extensively to increase mammary-tumor incidence (*4*), was employed. All offspring of experimental and control BALB/c mice were removed from the pens within a week after birth, so that only short periods of lactation intervened between gestations. Each female had at least 3 litters in rapid succession. These were recorded and, although the intensive breeding was continued throughout the period of observation, the number of litters that each bore after the third was not known.

All mice, including normally bred mice of the 5 strains, and force-bred BALB/c mice, both controls and fostered, were examined weekly for mammary tumors. The tumor age (the age of the animal when a tumor was palpable) was recorded and, after an interval of not more than 2 weeks, the mouse was killed and autopsied. Mice without grossly visible tumors were killed if death seemed imminent and were examined for nodules in the mammary-gland regions. All suspicious nodules were

fixed and prepared for histologic examination. All tumors were diagnosed histologically and classified as by Dunn (*10*).

OBSERVATIONS

Very few mammary tumors were observed in normally bred animals of strain BALB/c. There were 107 females in the colony during the period of fostering and, of these, 6, or 5.6 percent, developed tumors at a mean age of 15.7 months. Fifty-seven of the BALB/c females in the breeding colony bore the young that were fostered, and could have suckled them for a brief period before they were given to foster mothers. Three of these 57 females had tumors at 7.6, 20.6, and 22.1 months of age. Tumors in strain BALB/c mice were thus infrequent and usually occurred in rather old mice.

There were more tumors and they developed earlier in mice of the 4 high-tumor strains observed over the same period: 94.1 percent in strain C3H, at an average age of 8.9 months; 76.4 percent in strain A, at an average age of 11.8 months; 67.7 percent in strain DBA/1, at an average age of 14.6 months; and 64.5 percent in strain RIII at an average age of 11.1 months. Tumor incidences and average tumor ages among the foster mothers were not unlike those of all females in the breeding colonies, which indicated that the foster mothers were a good sample of the population. Sixteen of 17 (94.1%) strain C3H foster mothers had mammary tumors at a mean age of 9 months; 21 of 23 (91.3%) strain A foster mothers had mammary tumors at a mean age of 11.9 months; 16 of 21 (76.2%) strain DBA/1 foster mothers developed tumors at a mean age of 12.2 months; and 14 of 21 (66.6%) strain RIII foster mothers had tumors at a mean age of 10.8 months. None of the mice had mammary tumors at the time they were suckling the BALB/c young.

Table 1 summarizes the observations on mammary-gland tumors in the breeding colonies of strains BALB/c, C3H, A, DBA/1, and RIII; the

TABLE 1.—Mammary-tumor observations in normally bred mice of 1 low- and 4 high-tumor strains

Strain	Mice serving as foster mothers				Mice in breeding colonies			
	Mammary tumors				Mammary tumors			
			Age (*months*)				Age (*months*)	
	Number	Percent	Mean	Range	Number	Percent	Mean	Range
BALB/c	57*	5.3	16.8	7.6–22.1	107	5.6	15.7	7.6–22.1
C3H	17	94.1	9.0	6.1–13.1	170	94.1	8.9	4.6–18.3
A	23	91.3	11.9	6.8–13.4	127	76.4	11.8	5.7–21.2
DBA/1	21	76.2	12.2	7.6–21.6	164	67.7	14.6	5.4–29.0
RIII	21	66.6	10.8	6.7–21.6	155	64.5	11.1	5.8–22.3

*BALB/c natural mothers that may have suckled their young before these were given to foster mothers of the high-tumor strains.

strain BALB/c mothers of fostered animals; and the foster mothers from strains C3H, A, DBA/1, and RIII.

It was evident that our strain BALB/c was a low-tumor strain with infrequent mammary tumors, most of which occurred in old age. It was also evident that there were differences, both in incidence and mean tumor age, among our 4 high-tumor strains, C3H being conspicuous for the high percentage of tumors and the early age at which most of them occurred.

The observations on mammary tumors among the 4 groups of foster-nursed BALB/c females and the control BALB/c females are summarized in table 2, and in table 3 the fostered mice are compared with the strain from which the foster mother was selected. In general, the fostered mice were in the same order of tumor incidence and tumor age as the mice to which they were fostered—C_tC3H, like C3H, being conspicuous for high incidence and early age of mammary tumors. The C_tA, C_tDBA, and C_tRIII mice had more tumors and had them earlier than mice of the respective fostering strains, A, DBA/1, and RIII. Thus the genetic and hormonal constitution of the BALB/c mice was shown to be more favorable for mammary-tumor development than that of A, DBA/1, and RIII mice.

TABLE 2.—Observations on mammary tumors in 4 groups of foster-nursed strain BALB/c mice and unfostered controls, all force-bred

	Died with mammary tumors						Died without tumors		
				Age (*months*)				Age (*months*)	
Group	Number	Number	Percent	Mean	S.D.*	Range	Number	Mean	Range
C_tC3H	55	50	90.9	7.8	2.1	4.8–14.3	5	11.9	5.2–23.0
C_tA	61	54	88.5	9.3	3.2	4.9–19.7	7	13.0	6.3–21.2
C_tDBA	51	45	88.2	9.6	2.5	5.9–18.4	6	13.9	8.9–25.4
C_tRIII	53	42	79.2	10.7	3.3	6.2–19.7	11	15.6	6.3–24.9
All C_t	220	191	86.8	9.3	2.9	4.8–19.7	29	13.9	5.2–25.4
C†	78	4	5.1	19.6	2.1	17.6–22.0	74	19.8	7.9–35.6

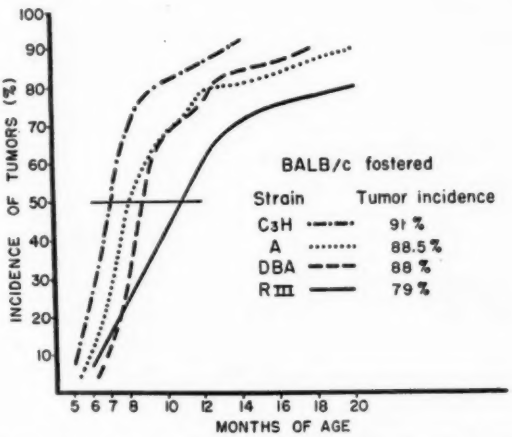
*S.D. is the standard deviation.
†BALB/c mice not fostered, but nursed by their own mothers.

TABLE 3.—Comparison of mammary tumors in foster-nursed strain BALB/c mice and in fostering strains

	Mammary tumors			Mammary tumors	
Fostered BALB/c	Percent	Mean age (*months*)	Fostering strain	Percent	Mean age (*months*)
C_tC3H	90.9	7.8	C3H	94.1	8.9
C_tA	88.5	9.3	A	76.4	11.8
C_tDBA	88.2	9.6	DBA/1	67.7	14.6
C_tRIII	79.2	10.7	RIII	64.5	11.1

Introduction of the agent with the foster mother's milk stimulated the development of tumors in a great proportion of BALB/c mice, but with no significant differences in incidence in the 4 groups. The greatest

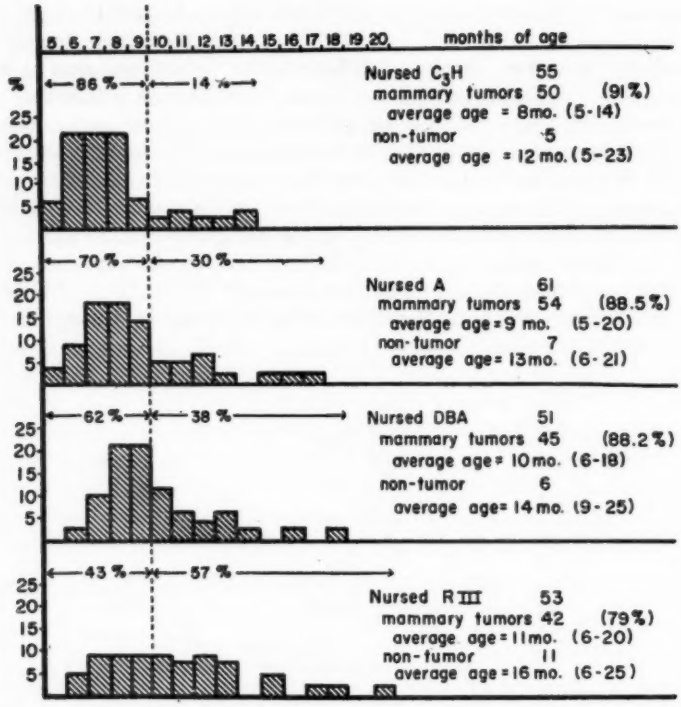
difference (11.7%) was between C_tC3H (90.9%) and C_tRIII (79.2%), and was not significant ($X^2 = 2.91$, $P = 0.10$–0.05), (*see* graph text-fig. 1). There were, however, significant differences in the average ages at which females of the different groups developed their mammary tumors. This is shown in the graph and also in the histogram, text-figure 2. Whereas 86 percent of the tumors of C_tC3H had appeared by the time these mice reached 10 months of age, 70 percent of the tumors of C_tA, 62 percent of the tumors of C_tDBA, and only 43 percent of the tumors of C_tRIII had become palpable at the same age. The C_tC3H group differed significantly from the C_tRIII group ($t=5.12$, $P<0.01$), from the C_tA group ($t=2.76$, $P< 0.01$), and from the C_tDBA group ($t = 3.93$, $P< 0.01$). C_tDBA and C_tA, and C_tDBA and C_tRIII did not differ significantly, though C_tA and C_tRIII did ($t = 2.08$, $P < 0.05$).



TEXT-FIGURE 1.—Graph comparing 4 groups of foster-nursed BALB/c mice with respect to incidence of mammary tumors at each month of age.

On the graph (text-fig. 1), the percent incidence of mammary tumors is plotted at monthly age intervals for each group of fostered mice. The mean ages at which the groups had tumor incidences of 50 percent were quite different, as shown on the graph, and the slopes of the curves indicate that there was a slower onset of the disease in C_tRIII mice than in the other groups. Whereas 50 percent of the C_tC3H mice had tumors at 7 months, and 50 percent of C_tA and C_tDBA at 8.25 and 8.75 months of age, the C_tRIII reached a mean age of 10.7 months before 50 percent had mammary tumors.

A difference in virulence might have been reflected also in the number of tumors per affected animal and in the histologic types. In number of tumors per animal, 85 percent of the C_tRIII mice had only one tumor, contrasted with 68 percent of the C_tC3H, 61 percent of the C_tA, and 57 percent of the C_tDBA. The differences were significant between C_tRIII and C_tA ($X^2 = 7.07$, $P < 0.01$), and C_tRIII and C_tDBA ($X^2 = 8.28$,


5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, months of age
%
86 %
14 %
Nursed C3H 55
mammary tumors 50 (91 %)
average age = 8mo. (5-14)
non-tumor 5
average age = 12 mo. (5-23)
70 %
30 %
Nursed A 61
mammary tumors 54 (88.5 %)
average age = 9 mo. (5-20)
non-tumor 7
average age = 13mo. (6-21)
62 %
38 %
Nursed DBA 51
mammary tumors 45 (88.2 %)
average age = 10mo. (6-18)
non-tumor 6
average age = 14 mo. (9-25)
43 %
57 %
Nursed RIII 53
mammary tumors 42 (79 %)
average age = 11mo. (6-20)
non-tumor 11
average age = 16 mo. (6-25)

TEXT-FIGURE 2.—Histogram comparing the 4 groups with respect to percent of tumors developing at each month of age and percent of total tumors developing before and after the females had reached 10 months of age.

$P < 0.01$), but not between other groups. No animal in the C_1RIII group had more than 2 tumors, none in the C_1DBA had more than 3 tumors, while 1 C_1C3H female and 3 C_1A females had 4 tumors apiece. Most of the tumors were adenocarcinomas of type B, which Andervont and Dunn (*10*) found to be characteristic of the mammary tumors of mice of strain BALB/c with the agent. In the C_1C3H group, 92 percent were type B; in the C_1A, 96 percent; in the C_1DBA, 91 percent; and in the C_1RIII, 86 percent.

DISCUSSION

Differences in mammary-tumor incidence and average tumor age have been attributed not only to the genotype of the strain transmitting the agent but also to the activity, concentration, quantity, and mutant forms of the milk-agent virus. Most of the studies of reciprocal F_1 hybrids between 2 high-tumor strains have reported differences in virulence of the agent transmitted by the 2 parental strains. Other experi-

ments have utilized cross-fostering techniques or compared mammary tumors induced by inoculation of the agent in extracts of tumors or normal tissues. All experiments have suggested strain differences in the agent and also substrain or subline differences in colonies that have been separated from each other for some period of time.

Bittner (*11*) crossed strains A and D (DBA) and found that the hybrids with strain A mothers had more tumors at younger ages than the reciprocal hybrids. Murray (*12*) reciprocally crossed mice of the Marsh and DBA strains, while Warner, Reinhard, and Goltz (*13*) used strains Marsh and A from the same laboratory. In each instance, the agent transmitted by the Marsh strain induced tumors at a lower rate. While Bittner and Huseby (*14*) found that hybrid virgin females of a cross between strains A and C3H had a higher incidence and a lower average age if they received the milk from strain A mothers, Heston and Andervont (*15*), in F_1 hybrid virgins between the same 2 strains, found a suggestion that the agent from strain C3H mothers was the more virulent. However, as Bittner (*4, 16*) and Andervont (*17*) have both emphasized, their C3H strains have been separated for some time and should be regarded as separate sublines. Recently, Richardson and Hummel (*18*) reported a difference in reciprocal F_1 hybrid virgins between strains C3H and RIII, the hybrids with the C3H mothers having a higher tumor-development rate than those with the RIII mothers.

Bittner (*16*) fostered agent-free C3H mice on strain A mothers and compared their tumor development with his and Andervont's sublines of C3H mice. The C3H-fostered strain A subline had tumors at an older average age than the others, though they were not greatly different in incidence. Blair (*6*) described a new strain of the milk agent or Bittner virus that induced early development of mammary tumors in a subline of strain A mice. She reported that the two agents in strain A mice had remained stable through several generations after cross-nursing and hybridization experiments.

Mühlbock (*19*) compared the strengths of the mammary-tumor agent in extracts of tumor tissue from 5 different high-tumor strains, and reported tumor tissue of strain A to be inferior to all others (DBA, C3H, RIII, and "White label") in transmittal of the agent. Using dried tumor tissue, Dmochowski (*20*) found the "tumour producing potency" of RIII tumor tissue to be greater than that of C3H tumor tissue, while that of strain A tumor tissue was the least effective. Barnum, Ball, and Bittner (*21*), comparing extracts of lactating mammary gland from mice of strains A and C3H, found that strain A lactating gland gave a high incidence at a higher dilution than strain C3H lactating gland.

The substrains of C3H and A in our experiment were separated from those of Heston and Andervont by only a few generations. This fact probably accounts for the difference in relative potency of the agents transmitted by strains C3H and A, as reported here and from Bittner's laboratory (*14, 16, 21*). Whereas the agent in Dmochowski's substrain of RIII was more potent than that of either his C3H or A strains, the

agent in the Jax substrain of RIII was less virulent than that of C3H, as shown by our data and those of Richardson and Hummel (*18*). Such differences in agent virulence in different mouse colonies can be explained by postulating the existence of strains of milk-agent virus. Blair's discovery of a mutant strain of the agent gave definite proof that the milk agent is like other viruses, with forms exhibiting different virulences.

Data in these foster-nursing experiments showed clearly that the agent in the milk of strain C3H mice differed from that in the milk of the other high-tumor strains in its ability to stimulate tumor development in strain BALB/c mice. The agents in the milk of strains A and DBA/1 did not exhibit different tumor-inducing rates in BALB/c mice, but there was a slight suggestion that the agent in the milk of strain RIII mice was less virulent. The C_tRIII mice had an older average tumor age than either C_tC3H or C_tA, and fewer tumors per animal than either C_tA or C_tDBA. The tumors in all BALB/c mice were similar in histologic type, which indicated that the constitution of the BALB/c was the factor determining the tumor type.

REFERENCES

(*1*) Bittner, J. J.: Mammary tumors in mice in relation to nursing. Am. J. Cancer 30: 530–538, 1937.

(*2*) Green, R. G., Moosey, M. M., and Bittner, J. J.: Antigenic character of cancer milk agent in mice. Proc. Soc. Exper. Biol. & Med. 61: 115–117, 1946.

(*3*) Heston, W. E., Deringer, M. K., Dunn, T. B., and Levillain, W. D.: Factors in the development of spontaneous mammary gland tumors in agent-free strain C3Hb mice. J. Nat. Cancer Inst. 10: 1139–1151, 1950.

(*4*) Bittner, J. J.: Inciting influences in the etiology of mammary cancer in mice. *In* Am. Assoc. Adv. Sc. Research Conference on Cancer (Moulton, F. R., ed.). Washington, D.C., Am. Assoc. Adv. Sc., 1945, pp. 63–96.

(*5*) Dmochowski, L.: Mammary tumour inducing factor and genetic constitution. Brit. J. Cancer 2: 94–102, 1948.

(*6*) Blair, P. B.: A new strain of the mouse mammary tumor virus. Science 127: 518, 1958.

(*7*) Andervont, H. B.: The influence of foster nursing upon the incidence of spontaneous mammary cancer in resistant and susceptible mice. J. Nat. Cancer Inst. 1: 147–153, 1940.

(*8*) Carter, T. C., Dunn, L. C., Falconer, D. S., Grüneberg, H., Heston, W. E., and Snell, G. D.: Standardized nomenclature for inbred strains of mice. Cancer Res. 12: 602–613, 1952.

(*9*) Bagg, H. J.: Further studies on the relation of functional activity to mammary carcinoma in mice. Am. J. Cancer 27: 542–550, 1936.

(*10*) Andervont, H. B., and Dunn, T. B.: Attempt to detect a mammary tumor-agent in strain C mice by X-radiation. J. Nat. Cancer Inst. 10: 1157–1189, 1950.

(*11*) Bittner, J. J.: Tumor incidence in reciprocal F_1 hybrid mice—A × D high tumor stocks. Proc. Soc. Exper. Biol. & Med. 34: 42–48, 1936.

(*12*) Murray, W. S.: Studies on the inheritance of mammary carcinoma in the mouse. Cancer Res. 1: 123–129, 1941.

(*13*) Warner, S. G., Reinhard, M. C., and Goltz, H. L.: The relative importance of the milk agent and inherited susceptibility to it in the development of mammary tumors in mice. Cancer Res. 5: 584–586, 1945.

(14) Bittner, J. J., and Huseby, R. A.: Relationship of inherited susceptibility and the inherited hormonal influence in the development of mammary cancer in mice. Cancer Res. 6: 235–239, 1946.

(15) Heston, W. E., and Andervont, H. B.: Importance of genetic influence on the occurrence of mammary tumors in virgin female mice. J. Nat. Cancer Inst. 4: 403–407, 1944.

(16) Bittner, J. J.: Mammary cancer in C3H mice of different sublines and their hybrids. J. Nat. Cancer Inst. 16: 1263–1286, 1956.

(17) Andervont, H. B.: Spontaneous tumors in a subline of strain C3H mice. J. Nat. Cancer Inst. 1: 737–744, 1940.

(18) Richardson, F., and Hummel, K. P.: Mammary tumors and mammary gland development in virgin mice of strains C3H, RIII, and their F_1 hybrids. J. Nat. Cancer Inst. 23: 91–107, 1959.

(19) Mühlbock, O.: Comparison of the mammary tumor agent in tumor extracts of various high cancer strains of mice. Proc. Koninklijke Ned. Akad. v. Wetensch. "C" 54: 386–390, 1951.

(20) Dmochowski, L.: Comparative potency of the mammary tumour agent of mice of different genetic constitution. Brit. J. Exper. Path. 26: 267–269, 1945.

(21) Barnum, C. P., Ball, Z. B., and Bittner, J. J.: Partial separation of the mammary tumor milk agent and a comparison of various sources of the agent. Cancer Res. 7: 522–528, 1947.

Glycogen Content of Cell Suspensions Prepared From Massive Tissue Culture. II. Comparison of Fourteen Cell Strains After Long Cultivation *In Vitro*[1] [2]

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SUMMARY

Thirteen mouse-tissue cell strains and one cell strain derived from human epidermal tissue were examined for their glycogen content after rapid proliferation for years *in vitro*. Strain NCTC 721 of mouse-liver epithelial origin, which was found to synthesize glycogen several years ago, still has this capacity. Strain NCTC clone 1469 derived from strain 721 of mouse liver also synthesizes glycogen. About 2 percent of the cultures from this clone gave rise to hepatomas at the site of injection when the cultures were returned to mice of the strain from which 721 originated. One of the hepatoma epithelial cell lines obtained 4 years after return to culture *in vitro* attained no detectable glycogen content, and the hepatomas derived from the cultures on re-injection in the animal had no glycogen. The human-skin strain NCTC 2198 contained glycogen. This strain had been grown from the epidermis of a 53-year-old man. Seven cell strains derived from mouse connective tissue were negative for glycogen. Three clones derived from mouse mammary carcinoma were likewise negative for glycogen.—J. Nat. Cancer Inst. 23: 823–831, 1959.

IN A previous study (*1*) it was found that the strain NCTC 721 cells derived from mouse-liver epithelium (*2*) stored more than 20 times the glycogen found in mouse fibroblasts of connective-tissue origin that were grown for many years under similar conditions in tissue culture. Several questions were raised as a result of this study:

1. Would the liver cells continue to preserve the capacity to store glycogen after further long-term cultivation *in vitro*? Would other cells of epithelial origin, after long growth *in vitro*, likewise preserve the capacity to store glycogen?
2. Was the lack of capacity of the fibroblast to accumulate glycogen characteristic of cells of connective-tissue origin? Was the lack of glycogen in the fibroblasts attributable to the fact that the fibroblast had undergone malignant change?

[1] Received for publication April 6, 1959.

[2] Part of this work was presented briefly at the meeting of the Tissue Culture Association in Atlantic City, N.J., April 10 to 12, 1959.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

3. Would the tumors rising from the injection of the tissue cells behave like the cultured cells with respect to forming and storing glycogen? This question arose partly because a culture strain of cells derived from liver strain 721 was available both in culture and as tumors growing in mice.
4. Would the tumors growing in the animals, from injection of tissue-culture cells, interfere with glycogen storage in the intact liver of the host? Sparse data in the literature indicated that tumors originating *in vivo* reduced the glycogen content of the liver of the host.

METHODS AND MATERIALS

The cell strains used are described elsewhere (*2–9*). The strain number, years in culture, tissue source, and medium in which they were grown are shown in table 1. The cells were grown in T-60 flasks with the nutrient fluid changed at thrice weekly intervals. For part of the study cells were harvested 48 hours after fluid change. For certain studies cells were harvested 4 hours after fluid renewal. The shorter interval was used because it had been found several years ago that the glycogen content of strain 721 cells (*1*) varied with the interval after feeding. Glycogen, after isolation from the tissues, was estimated by the anthrone reagent (*10–15*). For the isolation of glycogen from the tissues the following procedure was used: Cells in the T-60 flasks were shaken vigorously with the medium and poured into weighed centrifuge tubes. The cells were then packed lightly under a force of 80 × g in the centrifuge. This force appears to be about the maximum that the cells can sustain and still give good growth on resuspension in stock medium. The supernatant fluid was decanted and the cells were washed once with balanced saline, lacking glucose. The cells were then weighed and an aliquot taken for protein estimation by the Lowry procedure (*16*). The cells were suspended in 10 times their volume of 5.4 N KOH and placed in boiling water for 30 minutes. Twice the cellular volume of aqueous saturated sodium sulfate was added and 15 times the cellular volume of ethanol; after standing 30 minutes the precipitate was thrown down by centrifugation. The supernatant fluid was discarded and the tubes inverted to drain. The glycogen was dissolved in 0.25 N trichloroacetic acid. The tubes were again centrifuged to remove any undissolved precipitate, and the fluid was saved. Glycogen was precipitated from the trichloroacetic acid solution by the procedure of Carroll *et al.* (*12*). The precipitate was taken up in water and the anthrone reagent used as applied by Carrol. As a check on the method, glycogen was prepared from pooled livers of well-fed mice of the C3H/HeN line with the procedure of Abdel-Akher and Smith (*17*) or of Somogyi (*13*). The analytical procedure outlined was compared with that of Good *et al.* (*14*) and Carroll *et al.* (*12*). The solubility of glycogen is such, in the alkaline-alcohol mixture used by the Good procedure and by us, and in the

acid-alcohol extraction of the Carrol procedure, that recoveries of glycogen put through the procedures were always low. What this means from the practical standpoint is that if the total quantity of glycogen from any one tube were less than 20 micrograms it would have been scored as zero. Again what this means in terms of the quantity used for the cultured cells, *e.g.*, 100-mg. wet tissue cells per sample, is that the glycogen content could have been as much as 0.1 gm. per 100-gm. protein and still appear as zero in the final figures.

TABLE 1.—Cell strains used for study of their glycogen content

NCTC strain number	Derivation of cell strain or tissue	Years in culture*	Medium used for culture
721	Liver epithelium	10	S†
1469	One cell of 721	(10) 6	S
1795	Tumor from 1469	(6) 4	S
929	One cell of L	(18) 10	S
2168	929	(10) 2	NCTC 108 + 0.25 percent horse serum
2071	929	(10) 3	NCTC 108
1742	Connective tissue	8	S
1745	One cell of tumor from 1742	7	S
2049	Connective tissue	8	S
2050	One cell of tumor from 1742	7	S
2442	One cell of mammary carcinoma	7	S
2443	One cell of mammary carcinoma	7	S
2444	One cell of mammary carcinoma	7	S
2198	Epidermis of 53-year-old man	3	S

*Figures in parentheses are the years in culture of the parental strain of cells. All strains except 2198 were from the C3H/HeN mouse.

†S = Stock medium: 40 percent horse serum, 20 percent filtered chick-embryo extract, and 40 percent balanced saline.

When tissue directly from the mouse was analyzed, the animals were killed by cervical dislocation, and the tissue quickly excised, weighed, and placed in the KOH solution. The same procedure was applied as for the cultured cells. Wet and dry weights of the tissue were ascertained as previously described (*1*). As a qualitative test for glycogen, the method of Sumner and Somers was used (*18*).

RESULTS

Results are summarized in tables 2 to 5. Because various tissues, in addition to the cells taken directly from culture, were analyzed, the results are summarized in 5 sections:

I. Comparison of Glycogen Content of Liver From Normal Mouse, Liver From Sarcoma-Bearing Mouse, and Liver Cells in Culture

Livers from strain C3H/HeN mice were used. The mice had had free access to food and water, and were killed in midmorning. The sarcomas used arose at the site of injection of cells of the fibroblast strain 2050.

The liver cell strains from culture were those listed at the top of table 1. Table 2 lists the results. Glycogen content is expressed in gm. per 100 gm. of cellular protein. At 48 hours after fluid renewal, the cultured cells of strains 721 and 1469 have low glycogen and strain 1795 has no glycogen.

TABLE 2.—Glycogen content of mouse liver and cells derived from mouse liver grown in tissue culture for up to 10 years. Cells from culture harvested 48 hours after nutrient fluid change

Cell strain or tissue	Number of samples	Dry weight (*percent*)	Protein (*percent*)	Gm. glycogen per 100 gm. protein
Liver, normal C3H/HeN mouse.....	10	34. 7	21. 6	9. 7
Liver, sarcoma-bearing C3H/HeN mouse*.....	11	28. 6	22. 0	8. 2
721 cells.....	12	17. 7	11. 5	0. 17
1469 cells.....	12	17. 5	11. 5	0. 12
1795 cells.....	12	17. 5	11. 5	0. 0

*These sarcomas arose at site of injection of cultured cells of strain NCTC 2050, of table 1.

II. Influence of Type of Medium and Interval After Nutrient Fluid Renewal on Glycogen Content of Cultured Cells Derived From Liver

The three strains of liver origin were grown in a medium containing NCTC-109 solution plus 10 percent horse serum. Comparison was made of the glycogen content of the liver strains 4 hours after fluid renewal when they were grown in the latter medium and in the stock medium of table 1. The results are summarized in table 3. Four findings are

TABLE 3.—Influence of type of medium on glycogen content * of three cell strains of liver origin. Harvesting time, 4 hours postfluid-renewal

Cell strain	Grown in stock medium†	Grown in NCTC 109 plus 10 percent horse serum
721	3. 5	0. 4
1469	2. 2	0. 05
1795	0. 0	0. 0

* Glycogen content as gm. per 100 gm. of cellular protein.

†This is stock medium of table 1. Medium containing 10 percent horse serum is the equivalent of that used in table 1 for the sustenance of strain 2071 lacking protein, except that NCTC 108 has been replaced by NCTC 109 and adjustment made in the saline volume so that the constituents of NCTC 109 mixture are not diluted.

apparent: (*a*) Under experimental conditions used with these cell strains, nutrient medium consisting of NCTC 109 and 10 percent horse serum is not as effective for deposition of glycogen as the stock medium; (*b*) strain 721, after 10 years of cultivation *in vitro* in the stock medium, under these optimal conditions, stores considerable quantities of glycogen; (*c*) single cell strain 1469, derived from strain 721, likewise stores glycogen; (*d*) strain 1795 has lost this attribute.

III. Glycogen Content of Eleven Other Cell Strains of Table 1

The glycogen content of 7 cell strains of fibroblast origin, 3 cell strains of mouse mammary carcinoma origin, and the human epidermal strain is shown in table 4. The glycogen content of these strains was not influenced much by length of time after fluid renewal; none of the mouse strains stored glycogen under any conditions of culturing, and the human epidermis did not respond to short-term fluid renewal as strains 721 and 1469. However, the cells did not deplete stores of glycogen in 48 hours as much as the liver cells did.

TABLE 4.—Glycogen content of cells of various strains 48 hours after nutrient fluid change

Cell strain or substrain	Number of samples	Dry weight (*percent*)*	Protein (*percent*)*	Gm. glycogen per 100 gm. protein
2071	14	12.0	10	0
2168	6	12.0	10	0
929L	15	15.2	11.5	0
1742	10	15.5	12.0	0
1745	10	15.5	12.0	0
2049	10	15.5	12.0	0
2050	10	16.0	12.0	0
2442	3	15.5	11.5	0
2443	3	15.5	11.5	0
2444	3	15.5	11.5	0
2198	12	15.5	10.2	6.1

*Standard deviation is 0.1. Percent dry weight and protein content vary somewhat with the time after nutrient fluid renewal and quantity of cells per ml. of nutrient fluid. The samples chosen for this table were those showing similar population densities. Under these circumstances dry weight and protein content vary little within one strain of cells.

IV. Glycogen Content of Tumors Originating in C3H Mice From Injection of Cell Strains

As indicated in the work already published from this laboratory (*3*, *4*, *7*, and *9*) or in the present report, all 7 mouse strains of fibroblast origin, in table 1, gave sarcomas when they were injected into the mouse strain of origin. Cells of the 3 clones from mouse mammary carcinoma, on reinjection, gave sarcomas. Strain 1795 originated from a tumor arising at the site of injection of strain 1469 and now uniformly gives hepatomas when the cultured cells are injected. Tumors from fibroblast strains 1745 and 2050 and strain 1795 were examined for their glycogen content

immediately on removal from the animal. The results are not tabulated since all were negative.

V. Glycogen Content of Livers of Mice Bearing Tumors Originating From Injected Tissue-Culture Cells

Young *et al.* (*19*) and Gruzdeva (*20*) have reported that the livers of mice bearing transplanted tumors deposit less glycogen than the livers of the normal controls. Young also reported that human patients with gastric cancer had defective hepatic glycogenesis (*21*). In the light of these reports, when the tumors from the injection of the tissue-culture cells were harvested, the livers of the animals were also examined for glycogen. The glycogen content from the livers of animals bearing sarcoma originating from strain 2050 cells has been listed in table 1. In table 5, the results are recalculated in more conventional fashion as percent of the wet weight of the livers, to compare with the results of Young and Gruzdeva. Similar data for animals bearing strain 1745 sarcomas and 1795 hepatomas are included. These tumors apparently do not greatly discomfort the animals since the host mice appeared lively and ate well. Their liver glycogen reflects the fact that they are relatively normal, as shown in table 5.

TABLE 5.—Glycogen content of livers from normal mice and from mice bearing tumors: Percent of wet weight

Normal mice	2.5*
Bearing Ehrlich ascites tumor	0.4*
Normal CWF mice	2.25†
Bearing Sarcoma 180	1.15†
Normal C3H/HeN mice	2.1
Bearing sarcoma from 2050 cells	1.8
Bearing sarcoma from 1745 cells	1.8
Bearing hepatoma from 1795 cells	1.9

*Data from (*20*).
†Data from (*19*).

DISCUSSION

This study gave only partial answers to the questions posed in the introduction. Strain 721 of liver cells still is able to store glycogen after 10 years *in vitro*. The cells of clone 1469 derived from strain 721 also store glycogen. Strain 1795, originating in culture from a tumor from cells of clone 1469, did not store glycogen; the human epidermal strain stored glycogen. None of the strains of fibroblast origin or the three strains of mammary carcinoma origin deposited glycogen *in vitro*. None of the tumors from injection of the cells into mice had detectable glycogen. The tumors produced had only a slight effect in depressing glycogen stores in the livers of the host. Insofar as one may infer from data in the literature, when a depressing effect has been found, it probably relates to the poor nutritional state of the animal rather than to a direct effect of the tumor.

Glycogen phosphorylase activity is reputed to be present in almost all normal mammalian tissues. However, greatly reduced phosphorylase activities have been reported for a solid tumor (*22*) and for some 8 different tumors examined by Nirenberg (*23*). He ascribed the failure of ascites tumor cells to store glycogen to a unique glycogen-storage disease: Even though they possessed the phosphorylase-activating enzyme they lacked a functional dephosphophosphorylase. All tumors examined by Nirenberg had a similar block in the hormonally linked chain of phosphorylase activation. His work would appear to indicate that tumor tissue should lack the function of glycogen storage. Whether one may infer that a tissue is normal, if it once had the function of glycogen storage and after 10 years in tissue culture retains the function, is a question that is not completely answered. Since strain 1469 stored glycogen, but also gave tumors in a low percentage of the injections into animals, glycogen storage as a criterion for the normal state becomes suspect. The loss of one certain enzyme system may not be characteristic of all tumor cells. A transplantable hepatoma has been reported as having glycogen in the transplants (*24*). Human epidermis is known to contain glycogen (*25, 26*) and the cultured cells still have this capacity after years *in vitro*. The finding that the skin cells have a greater glycogen content than liver cells 48 hours after fluid renewal may indicate that the skin cells are less well supplied with those enzymes concerned with the mobilization and use of glycogen than the cells of liver origin. The low glycogen content of strains 721 and 1469, 48 hours after fluid change, probably represents the fraction of glycogen firmly bound to the enzyme proteins (*15*) which is difficult to extract with trichloroacetic acid (*12*). Although we have no data bearing on the point, it seems probable that glycogen synthesis in the liver cultures is by way of uridine diphosphate glucose, as found by Leloir and Cardini for fresh liver tissue (*27*). The quantity of glycogen, at a given time, in any one culture of those cells able to store glycogen is dependent to some extent on the number of cells per ml. in the culture, *e.g.*, if there are relatively few cells in a given volume of nutrient, they will make less demand on the available glucose or other glycogen precursors than if there are many present. The result of this would be that, if the cells are examined at 48 hours, as was done in tables 2 and 4, a small population might contain greater glycogen per cell because all the glucose might not be exhausted from the medium and the cells would not have started to draw on the glycogen stores. In an effort to control the population variable, the cultures reported here had about 2 million cells per ml. of nutrient fluid.

REFERENCES

(*1*) Westfall, B. B., Evans, V. J., Shannon, J. E., Jr., and Earle, W. R.: The glycogen content of cell suspensions prepared from massive tissue culture: Comparison of cells derived from mouse connective tissue and mouse liver. J. Nat. Cancer Inst. 14: 655–664, 1953.

(2) EVANS, V. J., EARLE, W. R., WILSON, E. P., WALTZ, H. K., and MACKEY, C. J.: The growth *in vitro* of massive cultures of liver cells. J. Nat. Cancer Inst. 12: 1245–1265, 1952.

(3) EARLE, W. R.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(4) SANFORD, K. K., LIKELY, G. D., and EARLE, W. R.: The development of variations in transplantability and morphology within a clone of mouse fibroblasts transformed to sarcoma-producing cells *in vitro*. J. Nat. Cancer Inst. 15: 215–237, 1954.

(5) PERRY, V. P., EVANS, V. J., EARLE, W. R., HYATT, G. W., and BEDELL, W. C.: Long-term tissue culture of human skin. Am. J. Hyg. 63: 52–58, 1956.

(6) HOBBS, G. L., SANFORD, K. K., EVANS, V. J., and EARLE, W. R.: Establishment of a clone of mouse liver cells from a single isolated cell. J. Nat. Cancer Inst. 18: 701–707, 1957.

(7) EVANS, V. J., HAWKINS, N. M., WESTFALL, B. B., and EARLE, W. R.: Studies on culture lines derived from mouse liver parenchymatous cells grown in long-term tissue culture. Cancer Res. 18: 261–266, 1958.

(8) MCQUILKIN, W. T., EVANS, V. J., and EARLE, W. R.: The adaptation of additional lines of NCTC clone 929 (strain L) cells to chemically defined protein-free medium NCTC 109. J. Nat. Cancer Inst. 19: 885–907, 1957.

(9) SANFORD, K. K.: *In vitro* studies of mammary carcinoma of the C3H mouse. In preparation.

(10) DREYWOOD, R.: Qualitative test for carbohydrate material. Indust. & Engng. Chem. (Anal. Ed.) 18: 499, 1946.

(11) SEIFTER, S., DAYTON, S., NOVIC, B., and MUNTWYLER, E.: The estimation of glycogen with the anthrone reagent. Arch. Biochem. 25: 191–200, 1950.

(12) CARROLL, N. V., LONGLEY, R. W., and ROE, J. H.: The determination of glycogen in liver and muscle by use of anthrone reagent. J. Biol. Chem. 220: 583–593, 1956.

(13) SOMOGYI, M.: The solubility and preparation of phosphorus- and nitrogen-free glycogen. J. Biol. Chem. 104: 245–253, 1934.

(14) GOOD, C. A., KRAMER, H., and SOMOGYI, M.: The determination of glycogen. J. Biol. Chem. 100: 485–491, 1933.

(15) STETTEN, M. R., KATZEN, H. M., and STETTEN, D., JR.: A comparison of the glycogen isolated by acid and alkaline procedures. J. Biol. Chem. 232: 475–488, 1958.

(16) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275, 1951.

(17) ABDEL-AKHER, M., and SMITH, F.: The repeating unit of glycogen. J. Am. Chem. Soc. 73: 994–996, 1951.

(18) SUMNER, J. B., and SOMERS, G. F.: The water-soluble polysaccharides of sweet corn. Arch. Biochem. 4: 7–9, 1944.

(19) YOUNG, N. F., KENSLER, C. J., SEKI, L., and HOMBURGER, F.: Deposition of liver glycogen in normal mice and in mice bearing sarcoma 180. Proc. Soc. Exper. Biol. & Med. 66: 322–323, 1947.

(20) GRUZDEVA, K. N.: The influence of glycine upon the liver glycogen content in healthy mice and mice suffering from cancer under the influence of Medinal. Byull. Exper. Biol. & Med. 42: 1032–1034, 1956.

(21) YOUNG, N. F., ABELS, J. C., and HOMBURGER, F.: Studies on carbohydrate metabolism in patients with gastric cancer. Defective hepatic glycogenesis; effects of adreno-cortical extract. J. Clin. Invest. 27: 760–765, 1948.

(22) HADJIOLOV, A. A., and DANCHENA, K. I.: Phosphorylase activity of primary rat liver cancer. Nature, London 181: 547–548, 1958.

(23) NIRENBERG, M. W.: An enzymic defect in ascites tumor cells. Biochem. & biophys. acta 30: 203–204, 1958.

(24) EDWARDS, J. E., DALTON, A. J., and ANDERVONT, H. B.: Pathology of a transplantable spontaneous hepatoma in a C3H mouse. J. Nat. Cancer Inst. 2: 555–563, 1942.

(25) MONTAGNA, W.: The Structure and Function of Skin. New York, Academic Press, Inc., 1956, 356 pp.

(26) ROTHMAN, S.: Physiology and Biochemistry of the Skin. Chicago, Univ. Chicago Press, 1953, 741 pp.

(27) LELOIR, L. F., and CARDINI, C. E.: Biosynthesis of glycogen from uridin diphosphate glucose. J. Am. Chem. Soc. 79: 6340–6341, 1957.

511629—59——18

A Study of the Sensitivity of the Mouse Forestomach Toward Certain Polycyclic Hydrocarbons [1, 2]

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SUMMARY

The forestomach of C57BL mice proved to be highly sensitive to the tumorigenic action of polycyclic carcinogens. A single application of 2 drops of 1.5 percent 7,12-dimethylbenz[*a*]anthracene (DMBA) to the skin of 47 C57BL mice was followed by the appearance of lymphomas in 11 mice and papillomas of the forestomach in 13. Among 33 control animals painted with benzene, only 1 died with a lymphoma, while no stomach tumors were found. A single intragastric application of 0.5 mg. of either DMBA or 3-methylcholanthrene (MC) gave rise to a forestomach tumor incidence of 85 percent and 57 percent, respectively. Multiple applications totaling 4 or 8 mg. of either DMBA or MC produced papillomas in all 44 treated animals and carcinomas in 6. Among 27 mice receiving 4 or 8 mg. of benz[*a*]anthracene (BA) by stomach tube, 2 developed papillomas of the forestomach after 16 months. No tumors appeared among 29 mice similarly treated once with 0.5 mg. of BA. The high sensitivity of the forestomach of the mouse suggests the use of this tissue for testing weakly carcinogenic compounds.—J. Nat. Cancer Inst. 23: 833–839, 1959.

DURING PILOT studies of the effects of cigarette-smoke condensate on the mouse skin, we observed a significant number of papillomas in the forestomach of C57BL mice, which had been "painted" once with approximately 1.5 mg. of 7,12-dimethylbenz[*a*]anthracene (DMBA). This observation suggested that the forestomach might serve as a test site for screening weakly carcinogenic polycyclic hydrocarbons. To investigate this possibility, the effects of 3 orally administered carcinogens on the forestomach of C57BL mice were studied. Both the pilot study and subsequent investigation are reported here.

Tumors have developed in the forestomach of mice after the oral administration of polycyclic hydrocarbons (*1–3*), heated cholesterol (*4*), and diazoaminobenzene (*5*). Berenblum and Haran noted that the forestomach, like the skin, responded to cocarcinogenic stimuli (*6, 7*). They produced a high incidence of forestomach tumors by the oral administration of as little as 1.5 mg. of DMBA or 3-methylcholanthrene (MC).

[1] Received for publication April 9, 1959.

[2] This work was supported in part by a grant from the Tobacco Industry Research Committee.

Tumors were elicited by croton oil alone, suggesting that the forestomach was highly sensitive to carcinogenic agents.

MATERIALS AND METHODS

The backs of 47 C57BL mice were "painted" with 2 drops (approximately 0.1 ml.) of a 1.5 percent solution of DMBA (Eastman #5149) in benzene. Two weeks later, the mice were separated into 3 groups. Mice of group A were then painted twice weekly with about 0.1 ml. of an acetone solution of crude cigarette-tar condensate (diluted to yield 12 ml. per carton of smoked cigarettes); group B mice were painted with acetone alone; group C remained without further treatment. Thirty-three mice (groups D and E) were "painted" with benzene instead of the DMBA solution, to serve as controls for groups A and B. Table 1 shows the distribution of mice in each group.

TABLE 1.—Distribution of tumors after a single painting with DMBA

Group	Treatment		Number of mice	Number of mice with tumors at autopsy		
	DMBA	Cigarette-smoke condensate		Forestomach papillomas	Lymphomas	Skin*
A	+	+	16	7	3	1
B	+	−	15	3	3	3
C	+	−	16	3	4	1
D	−	+	15	0	0	0
E	−	−	18	0	1	0
Total DMBA-treated mice			47	13	11	5
Total mice not treated with DMBA			33	0	1	0

*One mouse of group A and 2 mice of group C developed papillomas which regressed before autopsy.

At death, routine autopsies were performed. After gastric papillomas had been noted in several experimental animals, the intact stomachs from all subsequent autopsies were inflated with formalin and examined after fixation. The 15 experimental and 23 control animals still alive 524 days after the DMBA treatment were killed.

To determine the direct effect of carcinogens on the forestomach, C57BL mice were deprived of food for 24 hours, and then intubated through the esophagus with a polyethylene tube (O. D. 0.050″) attached to a hypodermic needle. Each animal received 0.1 ml. of heavy mineral oil containing 0.5 mg. of test compound. Repeated doses were given to some at intervals of 3 or 7 days. Table 2 shows the compounds tested, number of animals used, and dose schedules.

The animals were maintained in the laboratory without further treatment until gastric papillomas were found consistently among the animals that died. This required a period of 8 to 10 months for the DMBA or MC groups. Animals in the benz[*a*]anthracene (BA) series were killed after 16 months, although tumors had not been found in the *post-mortem*

material available. The mineral-oil controls were also killed after 16 months.

RESULTS AND DISCUSSION

Table 1 presents the data of the skin-painting experiment and shows that significant differences between groups depended only upon the DMBA treatment. DMBA apparently caused skin tumors, forestomach papillomas, and lymphomas. Forestomach tumors have been observed, after skin painting, by several other workers (*8–10*). These tumors probably developed because the animals licked the skin of their treated cage mates.

Table 2 presents the data of the oral-intubation experiment. BA produced tumors in only 2 of 27 animals given total doses of either 4.0 or 8.0 mg. of the hydrocarbon. A single dose of 0.5 mg. of either DMBA or MC was sufficient to cause gastric papillomas in over 50 percent of the mice. Repeated doses totaling 4.0 to 8.0 mg. of either compound were sufficient to produce forestomach papillomas in all 44 animals and carcinomas in 6. These data confirm those of Berenblum and Haran (*6*) who obtained a substantial number of tumors after single or repeated oral application of carcinogens to C3H and Swiss mice.

TABLE 2.—Production of forestomach tumors by oral administration of polycyclic hydrocarbons

Compound	Number of applications	Total dose (*mg.*)	Effective No. of animals	Mice with papillomas		Number with carcinomas
				Number	Percent	
DMBA	1	0.5	13	11	85	0
	8	4.0	17	17	100	2
	16	8.0	6	6	100	1
MC	1	0.5	14	8	57	0
	8	4.0	15	15	100	1
	16	8.0	6	6	100	2
BA	1	0.5	13	0	0	0
	8	4.0	19	1	5	0
	16	8.0	8	1	12	0
Mineral-oil controls	1		12	0	0	0
	8		16	0	0	0
	16		6	0	0	0

Two groups of investigators have painted the skin of C57BL mice with single doses of MC. Pullinger (*11*) applied 5 mg. of MC in acetone to the entire back, later removing a small piece of the skin to determine the effect of scarring. Of 46 mice surviving for 10 months, only 1 developed a skin tumor. Mider and Morton (*12*) found 7 skin papillomas in 28 C57BL mice that had been painted once with 0.5 percent MC. Comparison of their results with our intubation data indicates that the mouse forestomach is more sensitive to single applications of carcinogens than the skin. Even more convincing is the skin-painting experiment in which more gastric

papillomas than skin tumors developed, when the carcinogen was applied directly to the skin.

The high sensitivity of the forestomach is significant. Lipides are absorbed through the skin more readily than water-soluble compounds (*13*). The sebaceous glands seem to be involved in this process. Simpson and Cramer (*14*) demonstrated that methylcholanthrene entered the cells of the sebaceous glands within minutes of its application to the mouse skin. Suntzeff *et al.* (*15*) found that newborn mice, having only a few poorly developed sebaceous glands in the skin, were relatively resistant to the carcinogenic effect of methylcholanthrene painting. Similar results had been obtained by Lacassagne and Latarjet (*16*) in studies of mice painted with methylcholanthrene after scarring by intense ultraviolet irradiation. This evidence suggests that the sebaceous glands offer a direct and mandatory route for the penetration of this compound into deeper layers of the skin. In the stomach, however, no similar path exists. Nevertheless, enough hydrocarbon may enter the epithelial cells, during a single exposure, to provoke neoplastic change. In this case at least, a specific lipide pathway is not required.

Use of the mouse forestomach for screening carcinogens offers certain practical advantages. Basic information concerning the genesis of tumors of the forestomach is provided by Stewart and Lorenz (*17*). This tissue provides high sensitivity and is not subjected to additional trauma like the skin, in which wounds from shaving or fighting are not uncommon. The intubation technique requires neither a high level of skill nor much time.

However, with this method, gradual development of tumors cannot be observed. Identification of tumors is impossible unless dead animals are examined within a reasonable time. In addition, the method is not suitable for frequent applications of a test material. The data indicate that the forestomach is more sensitive than the skin and will give positive results with certain compounds that do not evoke skin tumors. Otherwise, the squamous epithelium of the stomach appears to give a response qualitatively similar to that of the skin (*6*, *7*, and *18*). Skin testing will probably remain a more convenient method for most materials, although the high sensitivity of the forestomach should be of value in testing weak carcinogens that are unavailable in quantity.

The first lymphomas caused by DMBA painting appeared in the thymus gland, after 122 days, while those which developed later involved the lymph nodes. One of the lymphomas was characterized by transplantable tumor cells in an abundant ascites fluid. When transplanted, most of the lymphomas killed the host within 3 to 6 weeks. While the C57BL mouse developed lymphomas after irradiation (*19*, *20*), development of this type of tumor after skin painting had not been observed previously (*21–23*). Since most reported experiments have been concerned with the painting of C57BL mice with other compounds, the development of lymphomas may be specific for DMBA. Lymphomas were not found in the intubated animals, perhaps because of differences in the route of administration.

REFERENCES

(1) LORENZ, E., and STEWART, H. L.: Squamous cell carcinoma and other lesions of the forestomach in mice, following oral administration of 20-methylcholanthrene and 1,2,5,6-dibenzanthracene. J. Nat. Cancer Inst. 1: 273–276, 1940.

(2) STEWART, H. L.: Hyperplastic and neoplastic lesions of the stomach in mice. J. Nat. Cancer Inst. 1: 489–509, 1941.

(3) SAXEN, E., EKWALL, P., and SETÄLÄ, K.: Squamous cell carcinoma of the forestomach in mice, following oral administration (cannula feeding) of 9,10-dimethyl-1,2-benzanthracene solubilized in an aqueous solution of an association colloid. Acta path. et microbiol. scandinav. 27: 270–275, 1950.

(4) WATERMAN, N.: Experimental production of carcinoma in the stomach of mice. Acta cancrol. 2: 375–388, 1936.

(5) OTSUKA, I.: Über die experimentelle Papillomerzeugung im Vormagen der Mäusen durch diazoaminobenzol. Gann 29: 209–212, 1935.

(6) BERENBLUM, I., and HARAN, N.: The influence of dose of carcinogen, emptiness of stomach and other factors on tumor induction in the forestomach of the mouse. Cancer Res. 15: 504–509, 1955.

(7) ———: The influence of croton oil and of polyethylene glycol-400 on carcinogenesis in the forestomach of the mouse. Cancer Res. 15: 510–516, 1955.

(8) BONNE, C.: Ueber Geschwülste bei Teertieren. Ztschr. Krebsforsch. 25: 1–22, 1927.

(9) TWORT, J. M., and TWORT, C. C.: Disease in relation to carcinogenic agents among 60,000 experimental mice. J. Path. & Bact. 35: 219–242, 1932.

(10) BADGER, G. M., COOK, J. W., HEWETT, C. L., KENNAWAY, E. L., KENNAWAY, N. M., MARTIN, R. H., and ROBINSON, A. M.: The production of cancer by pure hydrocarbons. V. Proc. Roy. Soc., London, ser. B 129: 439–467, 1940.

(11) PULLINGER, B. D.: An experimental approach to the problem of trauma and tumours. J. Path. & Bact. 57: 467–476, 1945.

(12) MIDER, G. B., and MORTON, J. J.: Relative importance of local and constitutional effects of methylcholanthrene in production of skin tumors in the mouse. J. Nat. Cancer Inst. 1: 41–44, 1940.

(13) ROTHMAN, S.: Physiology and Biochemistry of the Skin. Chicago, Ill., Univ. Chicago Press, 1954, p. 27.

(14) SIMPSON, W. L., and CRAMER, W.: Fluorescence studies of carcinogens in skin. I. Histological localization of 20-methylcholanthrene in mouse skin after a single application. Cancer Res. 3: 362–369, 1943.

(15) SUNTZEFF, V., CARRUTHERS, C., and COWDRY, E. V.: The role of sebaceous glands and hair follicles in epidermal carcinogenesis. Cancer Res. 7: 439–443, 1947.

(16) LACASSAGNE, A., and LATARJET, R.: Action of methylcholanthrene on certain scars of the skin in mice. Cancer Res. 6: 183–188, 1946.

(17) STEWART, H. L., and LORENZ, E.: Morbid anatomy, histopathology, and histopathogenesis of forestomach carcinoma in mice fed carcinogenic hydrocarbons in oil emulsions. J. Nat. Cancer Inst. 10: 147–166, 1949.

(18) KLEIN, A. J., and PALMER, W. L.: Experimental gastric carcinoma: A critical review with comments on the criteria of induced malignancy. J. Nat. Cancer Inst. 1: 559–584, 1941.

(19) HENSHAW, P. S.: Leukemia in mice following exposure to X-rays. Radiology 43: 279–285, 1944.

(20) KAPLAN, H. S.: Influence of age on susceptibility of mice to the development of lymphoid tumors after irradiation. J. Nat. Cancer Inst. 9: 55–56, 1948.

(21) KIRSCHBAUM, A., STRONG, L. C., and GARDNER, W. U.: Influence of methylcholanthrene on age incidence of leukemia in several strains of mice. Proc. Soc. Exper. Biol. & Med. 45: 287–289, 1940.

(22) MORTON, J. J., and MIDER, G. B.: Some effects of carcinogenic agents on mice subject to spontaneous leukoses. Cancer Res. 1: 95–98, 1941.

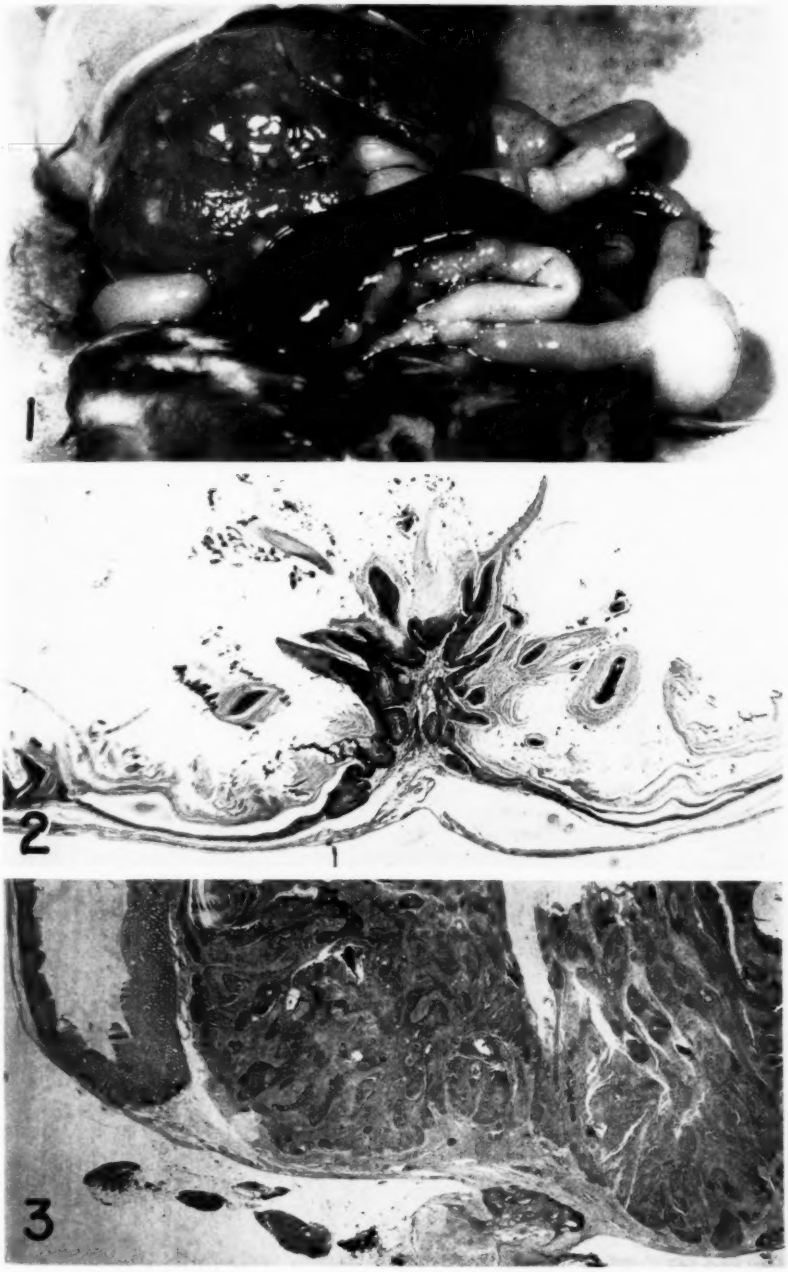
(23) KIRSCHBAUM, A., and MIXER, H. W.: Induction of leukemia in 8 inbred stocks of mice varying in susceptibility to the spontaneous disease. J. Lab. & Clin. Med. 32: 720–731, 1947.

PLATE 68

FIGURE 1.—Lymphoma in C57BL mouse 525 days after painting the skin with 2 drops of 1.5 percent 7,12-dimethylbenz[*a*]anthracene. In contrast to most of the lymphomas which appeared grossly to be limited to the thymus, this tumor involved the liver, spleen, and abdominal lymph nodes.

FIGURE 2.—Forestomach papilloma in the same mouse shown in figure 1.

FIGURE 3.—Carcinoma of the forestomach produced in 393 days by 16 intragastric applications of 0.5 mg. of 7,12-dimethylbenz[*a*]anthracene. Displacement of the glandular mucosa and penetration through the stomach wall can be noted.



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Topical Effect of Acriflavine Compounds on Growth and Spread of Malignant Cells [1, 2]

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SUMMARY

Routine tests of presumably antitumor agents, by assaying their ability to induce regression of tumor tissue and to increase the lifespan of the host, were supplemented by methods that had as test objectives (1) growth of free tumor cells in ascitic fluid, (2) implantation from this fluid into visceral and parietal peritoneum and adjacent tissues, and (3) spread and growth of experimentally infiltrated tumor cells in anatomically sharply circumscribed areas of the skin (the scalp and the scrotum), which allowed a quantitative estimate of these phenomena. Neutral acriflavine was selected for trial on account of (1) its antimetabolite effect against adenine-containing factors in bacteria and protozoa, which resulted in suppression of their viability; (2) its abundant storage in injected tissue at a therapeutic topical dose level; (3) its low systemic toxicity. The use of these methods demonstrated that acriflavine (1) inhibited the viability *in vitro* of tumor-cell suspensions after 2-hour incubation; (2) arrested completely, in well-tolerated doses, the proliferation of free tumor cells in ascitic fluid and their spread and implantation into peritoneal tissue; (3) prevented growth and spread of tumor cells that infiltrated into the scalp or the scrotum; and (4), in producing this effect, inflicted no visible damage to injected healthy tissue, *i.e.*, it affected selectively tumor cells. The significance of the testing methods used and the value of their results for the clinical application of acriflavine in prevention of tumor-cell spread in tissues are discussed.—J. Nat. Cancer Inst. 23: 841–855, 1959.

THE PRESENT trend in the search for therapeutic agents against malignant neoplasms is predominantly toward the screening of antimetabolites, since they offer better prospects of selective action than cytotoxic drugs (*1*). The choice of a substance is often motivated by its growth-inhibiting and metabolite-suppressing effects in cultures of microorganisms *in vitro* (*2*) or in bacterial infections *in vivo* (*3*). It is therefore rather surprising that acriflavine compounds were tested experimentally, for their effect on tumors, by only a few authors (*4*), and, generally, among numerous other agents screened by a single standard method of tumor inoculation and route of treatment, which was mostly

[1] Received for publication April 10, 1959.

[2] This project was supported by research grant DRG-466 from the Damon Runyon Foundation, and by research grant C-2080 (C5) from the National Cancer Institute, National Institutes of Health, Public Health Service.

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subcutaneous (*5–7*). The clinical assay of acriflavine was done mainly on infected tumors, usually of the breast and the bladder (*8, 9*), and therefore any improvement in the condition of the patients could be attributed at least partly to the strong antiseptic action of these compounds.

Acriflavines were discovered in 1909 by L. Benda (*10*) who was an associate of Paul Ehrlich, and were tested, by both authors, for their action on trypanosomes. In 1910 Werbitzki (*11*) reported that acriflavine hydrochloride (Trypaflavine) injected into a rat infected with trypanosomes was localized selectively on the blepharoplast (second nucleus); this cell damage may be responsible for trypanocidal effects. In 1917 Browning (*12*) recommended acriflavines for antibacterial therapy of wounds. In numerous publications on this subject the consensus was that acriflavines were of low toxicity, highly efficient in various bacterial and protozoal infections, and slowly absorbed from the site of injection (*3, 13*). Martin and Fisher (*2*) have reported that in microorganisms acriflavines inhibit physiologically important adenine-containing factors such as coenzymes I and II; these authors have reviewed the literature (up to 1943) on the interference of these compounds with bacterial metabolism, which may account for their antibacterial effects.

It seemed to us that storage of acriflavines at the site of injection, their selective damage to the cell nucleus in protozoa, and their effect on cell metabolism might suggest a trial of their local use against malignant cells. Accordingly, we have tested their ability to inhibit the growth and spread of tumor cells in tissues and exudates by topical application; these experiments are reported.

MATERIAL AND METHODS

Mice and tumor strains.—Male and female CFW mice (Carworth Farms, New City, N.Y.) and Swiss albino mice (Albino Farms, Red Bank, N.J.) weighing 24 to 27 gm. were used. Tumor strains Krebs-2 carcinoma and Sarcoma 180 were carried serially as ascites tumors in our laboratory.

Tumor-cell inoculation, withdrawal, and counts in the ascitic exudate.—Techniques of inoculation, of exudate withdrawal, and of cell counts in the exudate, as well as the method for calculation of tumor cell concentrations from total and differential counts, have been described in our early publications (*14–17*). The standard pattern of peritoneal implantation, periuterine localization, was induced. Approximately 10^7 or 10^6 tumor cells were inoculated whenever a massive growth was planned; in some preliminary experiments the dose was reduced to 10^5 to obtain a relatively low level of free tumor-cell concentration and implantation. The exudate was withdrawn for counts 3 or 4 days after inoculation; tissue specimens were taken for microscopic examination 6 to 72 hours after treatment of a 4-day-old growth.

Recording spontaneous implantation.—The majority of untreated control mice died within 10 days after an inoculation with a large number of

cells, and within 15 days after small inoculums. In those surviving longer, the implants were often necrotic. For this reason, the mice that had not died within 10 days were killed on the 10th day, and autopsies were performed. The extent of implant growth was graded as follows: 0 = no macroscopic implants; + = small nodules not more than 2 mm. in diameter; ++ = tumor 3 to 5 mm. in longer diameter; +++ = tumor larger than ++. The uniform pattern of periuterine localization and growth of implants provided a useful standard for an estimate of the extent of growth.

Induced implantation.—The abdominal wall of the mouse was divided into four quadrants and the peritoneum was punctured inside each quadrant with a 20-gauge needle. Twenty-four hours later, the animal was given an intraperitoneal inoculation (thus adding a fifth puncture) of tumor cells. When untreated animals were autopsied, large tumor nodules, with few exceptions, were found in the muscle beneath the peritoneum at the sites of punctures. Nodules up to 2 mm. in diameter were recorded as +; 3 to 5 mm., as ++; larger, as +++; absence of visible growth, as 0.

Experimental infiltration of subcutaneous tissue with free ascitic tumor cells.—We have reported previously (*16*, *18*) sharply localized growth of scalp tumors from free cell inoculums and their suitability for quantitative estimation of normal tissue invasion. In our present investigation we have used scalp tumors for testing the effect of an agent on the spread of subcutaneous malignant growth (*see* table 2). Ascitic exudate was inoculated into the scalp just above the eyes and the nose. The interval between the dates of inoculation and death was recorded. The extent of tumor spread was evaluated as follows: no growth, as 0; strictly localized growth on the top of the scalp, reaching orbits without involvement of the eyelids, as +; growth covering the whole area between the neck, the orbits involving the eyelids, and the ears, as ++; growth extension on the ears, the neck, and the face, as +++.

Extent of tissue invasion by free cells.—We have stated previously (*14*) that, in female mice, periuterine tissue invasion by tumor cells followed a standard pattern of localization which varied only quantitatively. For ascites tumor in male mice we have applied the following scale of tissue invasion, which was based on the extent of tumor-cell infiltration into the pancreas and into the loose connective tissue between the pancreas and the ventral surface of the spleen: no infiltration = 0; edematous connective tissue, groups of tumor cells outside and between the pancreas lobules = +; perisplenic tissue partly replaced by infiltrated tumor cells, either free or organized as tissue, widely separating the pancreas lobules = ++; perisplenic tissue mostly replaced by tumor encircling the pancreas lobules and the acini = +++; perisplenic tumor, malignant cells penetrating into the pancreatic acini, = ++++.

Intrascrotal inoculation and treatment.—A 23-gauge, long hypodermic needle punctured the skin at the junction of the upper and the lower left quadrants of the abdomen. It was introduced between the skin and the

peritoneum into the scrotal cavity, before injection of the tumor-cell suspension or a drug solution. In 92 percent of control animals a round tumor grew inside the scrotal wall (scale of estimated growth as previously mentioned) and eventually an ascites tumor occurred (*see* table 2).

Acriflavines.—Acriflavine hydrochloride and neutral acriflavine (Abbott Laboratories) were assayed comparatively in initial experiments; they showed no significant difference in activity, but the neutral compound was selected and used exclusively in the main experiments to avoid nonspecific factors such as slight acidity (*13*). The standard dose was 0.2 mg. intraperitoneally and 0.4 mg. subcutaneously, *i.e.*, 0.2 ml. and 0.4 ml. of a 1:1000 solution for mice weighing not less than 24 gm.; higher doses of 0.4 to 0.5 gm. were tolerated intraperitoneally if injected in a larger volume (2 cc.) of a higher dilution (1:10,000).

Controls to acriflavine.—In each of 3 series of experiments a group of 10 mice received 0.002 mg. of achromycin (Lederle) intraperitoneally or subcutaneously and acriflavine was given to experimental mice; negative results in controls showed that the topical effect of acriflavine could not be attributed to its antiseptic action on eventual latent infection.

Pattern of experiment.—In each series of experiments, acriflavine was given by a different route. In various groups of the same series, inoculations of graded doses of tumor cells were followed at various intervals (2 to 48 hours) by treatment with 2 to 4 daily injections of various doses of acriflavine, by the same route; thus, each experiment produced results of a different combination of 3 variants, *i.e.*, the amount of inoculum, the interval between the date of inoculation and the date of first treatment, and the schedule of treatment—doses given in each of the daily injections on successive days. Moreover, direct effect of acriflavine on tumor cells was assayed quantitatively by incubating graded doses of tumor cells in solutions of acriflavine of various concentration, in a volume of 2 ml. Control cell suspensions contained the same doses of tumor cells in 2 ml. of an 0.85 percent NaCl solution adjusted to the *p*H of the acriflavine solution, 6.5 or 6.6. All suspensions were shaken often and after 2 hours injected intraperitoneally into mice; 4 days later peritoneal fluid was withdrawn from these mice, smeared on slides, and stained by the aceto-orcein method (*15*); the scarcity or lack of tumor cells in specimens from experimental mice and the abundant growth (numerous mitoses) in controls were accepted as evidence of the inhibiting effect of acriflavine on tumor cells *in vitro*.

RESULTS

Peritoneal Growth

Proliferation of free tumor cells in the ascitic fluid, their implantation into the peritoneal wall, and their invasion of visceral connective tissue were inhibited by intraperitoneally injected solutions of neutral acriflavine. This phenomenon was studied in relation to 4 factors: tumor

strain, dose of inoculated tumor cells, stage of growth at the beginning of the treatment, and schedule of the treatment (number and doses of injections). Various quantitative combinations of these factors were more or less efficient in suppressing the growth and spread of tumor cells, as shown in table 1.

The data of table 1 suggested the following conclusions: (1) Use of different tumor strains, Krebs-2 and S-180, in similar experimental conditions (groups A1, A2, A3, and A4) was not associated with any wide differences in the results; thus, for two fast-growing tumor strains, the strain factor was unimportant. (2) Complete or significant inhibition of tumor-cell viability (figs. 1, 2, 3, and 4) was achieved only if the first treatment followed the inoculation by not more than 24 hours, *i.e.*, in the early stage of tumor growth (series A and B as compared with series C); thus, early treatment was a condition essential to complete inhibition. (3) Small doses of 0.2 mg. of acriflavine repeated 4 times resulted in higher tumor cell inhibition and were better tolerated than 2 high doses of 0.5 mg. Longer intervals (2 to 4 days) between 2 injections of 0.5 mg. did not increase but rather decreased the effect on tumor cells. Both efficiency and tolerance were increased by administration of acriflavine in a higher dilution—in 2 ml. instead of 0.2 or 0.5 ml., as in preliminary experiments. There was no damage to normal tissue in the injected area, but slight systemic symptoms, *e.g.*, lack of appetite and loss of weight, were induced by higher doses of 0.5 mg., probably as the result of the diffusion of acriflavine into the circulating blood. (4) These factors were screened against a standard large dose of tumor cells, 10^6, but in preliminary experiments with smaller inoculum, 10^5, complete inhibition was achieved even in the conditions of series A2 and A3. (5) All observations indicated that attenuation of tumor-cell viability in ascites tumors and tolerance of the mouse depended on the maintenance, obtained by repeated fractionated doses, of optimum acriflavine level in ascitic fluid; the effects on tumor cells within the range of our experiments were inversely proportionate to their concentration, which depended on the size of the inoculum and the age of its growth, in the same fluid.

Subcutaneous Growth

Tumors were implanted subcutaneously into the scalp, the scrotum, and the abdomen. Growth and spread (labeled together as "extension") of implants in the first two areas of treated and untreated animals are reviewed in table 2.

As in table 1, the experiments in table 2 were arranged for outlining the role of tumor factors (strain, size of the inoculum, and age of growth) and the treatment schedule (doses and number of injections) in the occurrence of quantitatively evaluated differences between treated mice and their controls in each group. The data obtained are analogous to those in peritoneal growth with the following differences: (1) a large (10^7) number of inoculated cells was completely inhibited by early (after

TABLE 1.—Growth and spread of tumor cells in mouse ascites tumors induced by inoculation of 10^6 tumor cells and treated at various intervals after inoculation with various doses of acriflavine*

Series and group	Tumor strain	Interval between inoculation and first treatment	Doses in 2 ml. distilled water (*mg.*); number of injections	Concentration of tumor cells in ascitic fluid (*thousands/mm.*^{3}) Averages		Peritoneal implantation in mice†		Visceral tissue (pancreas) invasion in mice‡	
				Controls	Treated	Controls	Treated	Controls	Treated
A1	Krebs-2	2 Hours	0.2 × 4	59.3	0	+++	+	++	0
A2	Krebs-2	2 Hours	0.3 × 3	54.4	0.27	+++	+	++++	+
A3	Krebs-2	2 Hours	0.5 × 2	64.4	0.45	+++	++	+++	+
A4	S-180	2 Hours	0.2 × 4	52.7	0.18	++	++	++	0
B1	Krebs-2	24 Hours	0.2 × 4	41.9	0.35	+++	++	++++	+
B2	Krebs-2	24 Hours	0.3 × 3	63.7	3.15	+++	+	+++	+
B3	Krebs-2	24 Hours	0.5 × 2	47.4	2.1	++	++	+++	0
C1	Krebs-2	72 Hours	0.3 × 3	51.6	38.9	+++	+++	+++	+++
C2	Krebs-2	72 Hours	0.5 × 2	57.0	41.7	+++	+++	+++	++++
C3	Krebs-2	72 Hours	0.2 × 4	49.4	52.2	+++	+++	++++	+++

*Results are based on 20 mice in each group; 16 were given neutral acriflavine and 4, acriflavine hydrochloride.

†Scale of peritoneal implantation: no growth = 0; nodule 1 to 2 mm. in diameter = +; 3 to 5 mm. = ++; larger = +++.

‡Scale of visceral tissue (pancreas) invasion: no infiltration = 0; group of tumor cells outside and between pancreas lobules = +; tumor cells widely separating pancreas lobules = ++; perisplenic tissue mostly replaced by tumor encircling pancreas lobules and acini = +++; perisplenic tumor, malignant cells penetrating into pancreatic acini = ++++.

TABLE 2.—Subcutaneous growth of implants in the scalp and the scrotum after peritumoral tissue infiltration with various repeated doses of acriflavine*

Series and group	Tumor strain	Interval between inoculation and first treatment (*hours*)	Doses of acriflavine and number of daily injections (*mg. in 0.5 ml.*)	Extension of tumor growth in mice		Mice survival after 30 days	
				Treated	Controls	Treated	Controls
Scalp implants†							
A1	Krebs-2	2	0.5 × 4	0	++++	19/1	0
A2	Krebs-2	2	0.3 × 4	0	++++	18/2	1
A3	S-180	2	0.5 × 4	+	+++	20/0	0
B1	Krebs-2	24	0.5 × 4	+	++++	16/4	0
B2	Krebs-2	24	0.3 × 4	+	++++	18/2	1
B3	Krebs-2	24	0.1 × 4	+	++++	15/5	0
C1	Krebs-2	72	0.5 × 4	+	++++	13/7	0
C2	S-180	72	0.5 × 4	+	++++	15/5	2
C3	Krebs-2	72	0.3 × 4	+	++++	12/8	0
Scrotum implants							
D1	Krebs-2	2	0.5 × 4	0	+++	18/2	1
D2	Krebs-2	2	0.3 × 4	0	+++	15/5	1
D3	S-180	2	0.5 × 4	+	++	17/3	2
E1	Krebs-2	24	0.5 × 4	+	+++	11/9	1
E2	Krebs-2	24	0.3 × 4	+	++	8/12	0
E3	Krebs-2	24	0.1 × 4	+	++	8/12	2
F1	Krebs-2	72	0.5 × 4	+	+++	5/15	0
F2	S-180	72	0.5 × 4	++	++	10/10	1
F3	Krebs-2	72	0.3 × 4	+	++	4/16	0

*Results based on 20 mice in each group. All animals were inoculated with 10^7 tumor cells.

†Extension of implant growth in the scalp was graded as follows: no growth = 0; strictly localized growth on top of scalp reaching orbits without involvement of eyelids = +; growth covering whole area between neck, orbits involving eyelids and ears = ++; growth extension on ears, neck, and face = +++.

2 hours) administration of average amounts, 0.3 mg. × 4, of acriflavine; (2) 3-day-old tumors regressed, however, with cicatrization in scalp implants (fig. 7) and necrotization in scrotum implants (fig. 9) after standard treatment (groups C1, C2, F1, and F2); (3) requisite doses of compound were injected in a volume of 0.5 ml. instead of 2 ml. for peritoneal cavity; (4) in preliminary experiments (unrecorded here), regression of 2-hour growth could be obtained occasionally with a single dose of 0.5 mg. in scalp tumors; in experiments (nontabulated) with subcutaneous tumors of the abdomen, incomplete regression in treated animals was often evidenced by tumor outgrowth from a small area on the periphery of the scalp surface or at the site of scrotal sac attachment; their regression occurred as intensive necrotization, often with infection, which made the results unsuitable for precise quantitative evaluation. Small, sharply delimited nodules in treated mice contrasted with diffuse growth and spread of tumor in controls and illustrated the effect of peritumoral treatment on both growth and spread of the inoculum.

In the experiments of table 3, tumor cells were placed in direct contact with acriflavine *in vitro* and their viability after the exposure was tested *in vivo* by intraperitoneal inoculation.

TABLE 3.—Viability assays by intraperitoneal injections of tumor cells after incubation in acriflavine solutions*

Series and group	Tumor strain	Approximate number of tumor cells inoculated in 0.2 ml. of acriflavine suspension	Concentration of acriflavine dissolved in tumor-cell suspension (*mg./ml.*)	Concentration of tumor cells in ascitic fluid in mice inoculated with tumor-cell suspension (*thousands/mm.*^{3}) Averages		Amount of implant growth in the peritoneum of mice inoculated with tumor-cell suspension†	
				In saline solution (*controls*)	In acriflavine solution	In saline solution (*controls*)	In acriflavine solution
A1	Krebs-2	10^6	1. 0	51. 7	0	++	0
A2	Krebs-2	10^7	1. 0	62. 3	0. 19	+++	+
B1	S-180	10^6	1. 0	55. 2	0	+++	0
B2	S-180	10^7	1. 0	49. 4	0. 29	++	+
C1	Krebs-2	10^6	0. 5	57. 6	4. 2	++	+
C2	Krebs-2	10^7	0. 5	64. 5	11. 7	+++	+
D1	Krebs-2	10^6	2. 0	51. 5	19. 8	+++	++
D2	Krebs-2	10^5	2. 0	37. 7	17. 5	++	++
E1	Krebs-2	10^6	0. 25	44. 5	4. 6	++	+
E2	Krebs-2	10^5	0. 25	29. 2	0. 14	++	+

*Results in inoculated mice based on 15 mice in each group. Neutral acriflavine used in all experiments.
†Amount of implantation graded as in table 1.

The data in table 3 are based on several experiments, each using the same sequence of acriflavine concentration. The results for each concentration were pooled and tabulated. The results could be summarized in a statement that the attenuation *in vitro* of tumor-cell viability was inversely proportionate to the concentration of tumor cells in their inoculum suspension. It should be emphasized that even large doses (10^6) of cells were inactivated in lower (1:2000) concentration of acriflavine (groups E1 and E2). Concentrated solutions of acriflavine (D1 and D2) induced clumping and inhibited viability of tumor cells less completely than in higher dilutions; it is probable that only cells on the surface of clumps were exposed to acriflavine. Clumping never occurred on a low dose level and, therefore, it could not be attributed to any other factor than high concentration. Clumping of bacteria in high concentrations of acriflavine was described by other authors (*3*, *12*). Scanty cells detected in ascitic fluid by microscopic examination (tables 1 and 3, series A and B; fig. 1) often showed lack of viability in bioassay, *i.e.*, after their transfer into the peritoneal cavity of new mice.

The possibility of action *in vivo* in acriflavine experiments of table 3 is entirely excluded, since a single injection of acriflavine even in higher doses never induced sizable inhibition of ascitic tumor-cell viability.

It should be also remembered that the effect *in vitro* of neutral acriflavine could not be attributed to the *p*H.

Implantation (nodules) of tumor cells from the ascitic fluid at the sites of abdominal punctures (see *induced implantation* in Materials and Methods) could not be induced in treated animals if the punctures were performed before tumor-cell inoculation. However, they occurred in areas punctured for daily injections of the compound (fig. 5). In untreated animals implantation occurred consistently at all sites of punctures, whether performed before or after inoculation (fig. 6). In other words, the treatment prevented implantation of tumor cells from the fluid into fibrin and connective tissue made available by the puncture, but not if tumor cells had access to the needle canal before reparative processes had set in. The significance of these findings will be discussed.

DISCUSSION

Woglom (*19*) stated that a "transplantable tumor although a malignant neoplasm is in itself, one of the most harmless things imaginable, exerting no evil effect until by ulceration it has become a portal of entry for bacteria, or until hemorrhage or metastasis has supervened." Thus, he emphasized that the "virulence" of experimental tumors, *i.e.*, their damaging effect on the host cannot be estimated in terms of death rate of tumor-bearing mice. By the same reasoning, reduction of their lethality, *i.e.*, extension of their lifespan after treatment with an agent, is an evidence of the "carcinolytic" property only if the antiseptic, antihemorrhagic, and other nonspecific effects were excluded. Lettré's (*6*) report

on the extended survival of a group of mice with Ehrlich's adenocarcinoma ascites tumor after treatment with acriflavine could be attributed, at least partly, to the effect of an eventual latent infection. Accordingly, it occurred to us that, for this purpose, routine tests of presumably antitumor agents, which were introduced years ago (*20, 21*), and used for assaying their ability to induce regression of tumor tissue and to increase the lifespan of the host, could be usefully supplemented by additional methods, accepting as a test object tumor cells growing free in the fluid and spreading by infiltration into tissues.

In the present investigation we have tested an acriflavine compound—neutral acriflavine— only topically, by injections into areas where previously inoculated cells had spread and initiated growth. This choice of route has been suggested by recent developments of topical chemoprevention and radioprevention of tumor-cell spread in normal tissues as more promising than systemic chemotherapy in some stages and manifestations of malignant disease (*18, 22, 23*). Thus, intracavitary treatment of peritoneal and pleural carcinosis with radioisotopes or nitrogen mustards has been universally accepted. Shimkin and Moore (*24*) have initiated as a part of their adjuvant chemotherapy program a planned trial of topical chemotherapy for prevention of tumor-cell spread from operative wounds into surrounding tissues and the blood. It seemed to us that our methods of chemoprevention, which are described and applied in the present investigation, may contribute to investigation of the problems introduced by the previously mentioned new orientation of clinical chemotherapy. A study of similar problems in rats has been reported recently by Druckrey *et al.* (*25*).

Our results *in vitro* in table 3 have demonstrated irreversible, at least in conditions of our experiments, disappearance of viability even in a large number (10^7) of tumor cells after their incubation in neutral acriflavine in concentrations well tolerated by mice (tables 1 and 2) and by humans (*3, 9, 12*). This effect of acriflavine is obviously specific and can be attributed to the mechanism responsible for its specific affinity for the protozoal nucleus (*11*) and its inhibition of some essential metabolites (*2*) in bacteria.

This complete inhibition of tumor-cell activities *in vitro* could be demonstrated also *in vivo* when the compound was injected into the peritoneal cavity (table 1) or subcutaneously (table 2) shortly after inoculation with a relatively high number of tumor cells (10^6). More copious inoculums (10^7) or delayed treatment, more than 24 hours after inoculation, resulted in only a partial inhibition. Accordingly, in ascites tumors, topical acriflavine injections produced significant preventive results and only moderate therapeutic effects.

In subcutaneous tumors, in particular those of the scalp, even a late peritumoral treatment arrested the spread of tumor cells (table 2; figs. 7, 8, 9, and 10).

The action of acriflavine on tumor cells was distinctly selective. No damage to normal tissues was produced in new mice by the doses and

concentrations used in our experiments. In treated tumor-bearing animals no gross changes were noted in normal tissues after disappearance of infiltrating tumor cells; after late treatment peritoneal and subcutaneous implants became frequently totally or partly necrotic, but their surrounding tissue was involved only by a low-grade inflammation and by cicatrization.

Tumor-cell suspensions in our experiments are inhibited in their growth by acriflavine in considerably higher concentration than bacterial cells in experiments of early authors (*3*, *12*); however, this quantitative difference in the effect may be explained by the higher ability of tumor cells, as more complicated and organized structures, to escape up to a certain level the effect of the antimetabolite by using alternate (substitute) metabolism. An analogous difference between little differentiated tumor cells and highly differentiated normal cells, implying still higher resistance of normal cells to antimetabolites by adoption of an alternate metabolic process, may account for the selected damaging effect of acriflavine, and perhaps of some other antimetabolites, on tumor cells.

Our results resemble the outcome of our experiments carried out by similar methods on preventive application of Thio-TEPA in ascites and subcutaneous tumors (*23*). However, Thio-TEPA had the advantage of more rapid action on tumor cells which disappeared almost completely within 24 hours after the first injection. In ascites tumor-bearing mice treated with Thio-TEPA, no implantation was found at the site of punctures made either before or after intraperitoneal inoculation, while acriflavine prevented implantation only if the peritoneum was punctured before inoculation (figs. 5 and 6). Acriflavine had the advantages of very low toxicity and of copious storage in the injected tissue. These advantages recommend a clinical trial of acriflavine for prevention of tumor-cell spread in peritumoral tissue, for inhibition of growth from already infiltrated cells, and for intracavitary therapy, perhaps as an adjuvant to already accepted agents.

CONCLUSIONS

1. A large number (10^6–10^7) of Krebs-2 or S-180 tumor cells, incubated for 2 hours in a 1:1000 solution of neutral acriflavine and injected intraperitoneally into new mice, failed to induce growth; thus, their viability was specifically and irreversibly inhibited by acriflavine. This phenomenon was attributed to inhibition, by acriflavine, of adenine-containing cell metabolites and to a selective damaging effect to the nucleus of some free cells, as described by several authors for bacteria and protozoa.

2. Daily intraperitoneal injections of 0.2 mg. of acriflavine in 2 ml. of water, starting 24 hours after inoculation of the mouse, arrested completely the proliferation of free tumor cells and their spread and implantation into peritoneal tissue of ascites tumors, Krebs-2 and S-180. This

inhibition was only partial (*a*) if lower doses of acriflavine, 0.1 mg., were given; (*b*) if longer intervals elapsed between inoculation and treatment; or (*c*) if a larger number (10^7) of tumor cells were inoculated.

3. Similarly the outcome of topical treatment, with the same doses and number of injections, of circumscribed skin areas (the scalp and the scrotum) infiltrated with tumor cells was directly proportionate to the dose of acriflavine, within the range used, and inversely to the interval between inoculation and treatment and to the size of the inoculum.

4. It was concluded from 2 and 3 that early topical administration of acriflavine had a significant preventive effect on proliferation and spread of tumor cells.

5. Late treatment of subcutaneous inoculum arrested the spread of proliferated tumor cells and often induced their necrosis with (the scalp) or without (the scrotum) cicatrization. The absence of gross damage to normal tissue in these and new mice after injections of acriflavine demonstrated the selective quality of its effect on tumor cells.

6. It is hoped that acriflavine may find application for the prevention of tumor-cell spread in tissues and in cavities, and that the methods used in this investigation may help to screen other agents for similar applications.

REFERENCES

(*1*) GALTON, D. A. G.: Clinical and biological aspects. Symposium on anti-tumor agents. Proc. Royal Soc. Med. 50: 10–12, 1957.

(*2*) MARTIN, G. J., and FISCHER, C. V.: Nutritional status of *Staphylococcus aureus* as influenced by proflavine. J. Lab. & Clin. Med. 29: 383–389, 1944.

(*3*) BROWNING, C. H.: The present status of aminoacridine compounds (flavines) as surface antiseptics. Brit. M. J. 1: 341–343, 1943.

(*4*) DYER, H.: An Index of Tumor Chemotherapy. Monogr. Washington, D.C., Nat. Cancer Inst., Federal Security Agency, 1949.

(*5*) DUSTIN, A. P., and GREGOIRE, M. C. H.: Contribution à l'étude de l'action des poisons caryoclasiques sur les tumeurs animales.—Premér mémoire: Action du cacodylate de Na et de la trypaflavine sur le sarcome greffé, type Crocker de la Souris. Bull. Acad. roy. de med. de Belgique 13: 585–592, 1933.

(*6*) LETTRÉ, H.: Zur Wirkung von Trypaflavin auf den Mause-Ascites-Tumor. Zeitschr. physiol. Chem. 135: 270–271, 1941.

(*7*) MARSCH, M. C., and SIMPSON, B. T.: Chemotherapeutic attempts with coal-tar derivatives in spontaneous mouse tumors. J. Cancer Res. 11: 417–437, 1927.

(*8*) BLUMENTHAL, F.: Chemotherapie des Krebses. Beitr. z. klin. Chir. 154: 50–73, 1931

(*9*) LEWIN, C.: Trypaflavin and trypaflavinsilber (argoflavin). Therapie maligner Geschwulste. Therapie der Gegenwart. 61: 10–12, 1920.

(*10*) BENDA, L.: *In* Paul Ehrlich's Festschrift. (Fischer, J. G.), 1914, p. 57.

(*11*) WERBITZKI, F. M.: Über Blepharoplastlose Trypanosomen. Cbl. f. Bakt. 1. Abt. Or. 53: 363–370, 1910.

(*12*) BENNETT, C., BLACKLOCK, J. W. S., and BROWNING, C. H.: The action of flavine antiseptics on localized pyogenic infections. Brit. M. J. 2: 306, 1922.

(*13*) OSOL, A., and FARRAR, G. E.: Acriflavine. U.S. Dispensatory, 24th ed. Philadelphia, J. B. Lippincott Company, 1947.

(14) GOLDIE, H.: Growth characteristics of free tumor cells in various body fluids and tissues of the mouse. Ann. New York Acad. Sc. 63: 711–727, 1956.

(15) GOLDIE, H., and FELIX, M. D.: Growth characteristics of free tumor cells transferred serially in the peritoneal fluid of the mouse. Cancer Res. 11: 73–80, 1951.

(16) GOLDIE, H., JEFFRIES, B. R., GREEN, S., and SIMPSON, M. R.: Primary growth of leukemic cells as a factor in their metastatic growth and in their lethal effect on the mouse. Cancer Res. 14: 423–427, 1954.

(17) GOLDIE, H., JEFFRIES, B. R., MAXWELL, M. C., and HAHN, P. F.: Growth of free tumor cells in the pleural exudate and their implantation into the pleura of the mouse. Cancer Res. 12: 422–425, 1952.

(18) GOLDIE, H., and WEST, H. D.: Effect of peritumoral tissue infiltration with radioactive yttrium on growth and spread of malignant cells. Cancer Res. 16: 484–489, 1956.

(19) WOGLOM, W. H.: Immunity to transplantable tumors. Cancer Rev. 4: 129–195, 1929.

(20) WASSERMANN, A. V., KEYSSER, F., and WASSERMANN, M.: Beiträge zum Problem Geschwülste von der Blutbahn aus therapeutisch zu beeinflussen. Auf Grund chemotherapeutischer Versuche an tumorkranken Tieren. Deutsche. med. Wchnschr. 2: 2389–2391, 1911.

(21) WEIL, R.: Chemotherapy and tumors. J.A.M.A. 64: 1283–1285, 1915.

(22) GOLDIE, H., and HAHN, P. F.: Distribution and effect of colloidal radioactive gold in peritoneal fluid containing free sarcoma 37 cells. Proc. Soc. Exper. Biol. & Med. 74: 634–642, 1950.

(23) GOLDIE, H., WALKER, M., MITCHELL, G. B., and GRAHAM, A. R.: Effect of aminoteropterin and of ThioTEPA on growth and implantation of free-tumor cells from ascites tumors. Cancer Res. 17: 374–381, 1957.

(24) SHIMKIN, M. B., and MOORE, G. E.: Adjuvant use of chemotherapy in the surgical treatment of cancer. J.A.M.A. 167: 1710–1714, 1958.

(25) DRUCKREY, H., KUK, B. T., SCHMAHL, D., and STEINHOF, D.: Kombination von Operation und Chemotherapie beim Krebs. München. med. Wchnschr. 100: 1913–1918, 1958.

PLATE 69

FIGURE 1.—Smear from ascitic fluid 8 days after inoculation of 10^6 tumor cells, 2 days after 4th daily injection of 0.2 mg. acriflavine.

FIGURE 2.—Smear from untreated mouse; control to figure 1.

FIGURE 3.—Peritoneal cavity of mouse inoculated and treated as in figure 1.

FIGURE 4.—Untreated mouse; control to figure 3.

FIGURE 5.—Abdominal wall of mouse punctured (on left side) before inoculation and treatment as in figures 1 and 3.

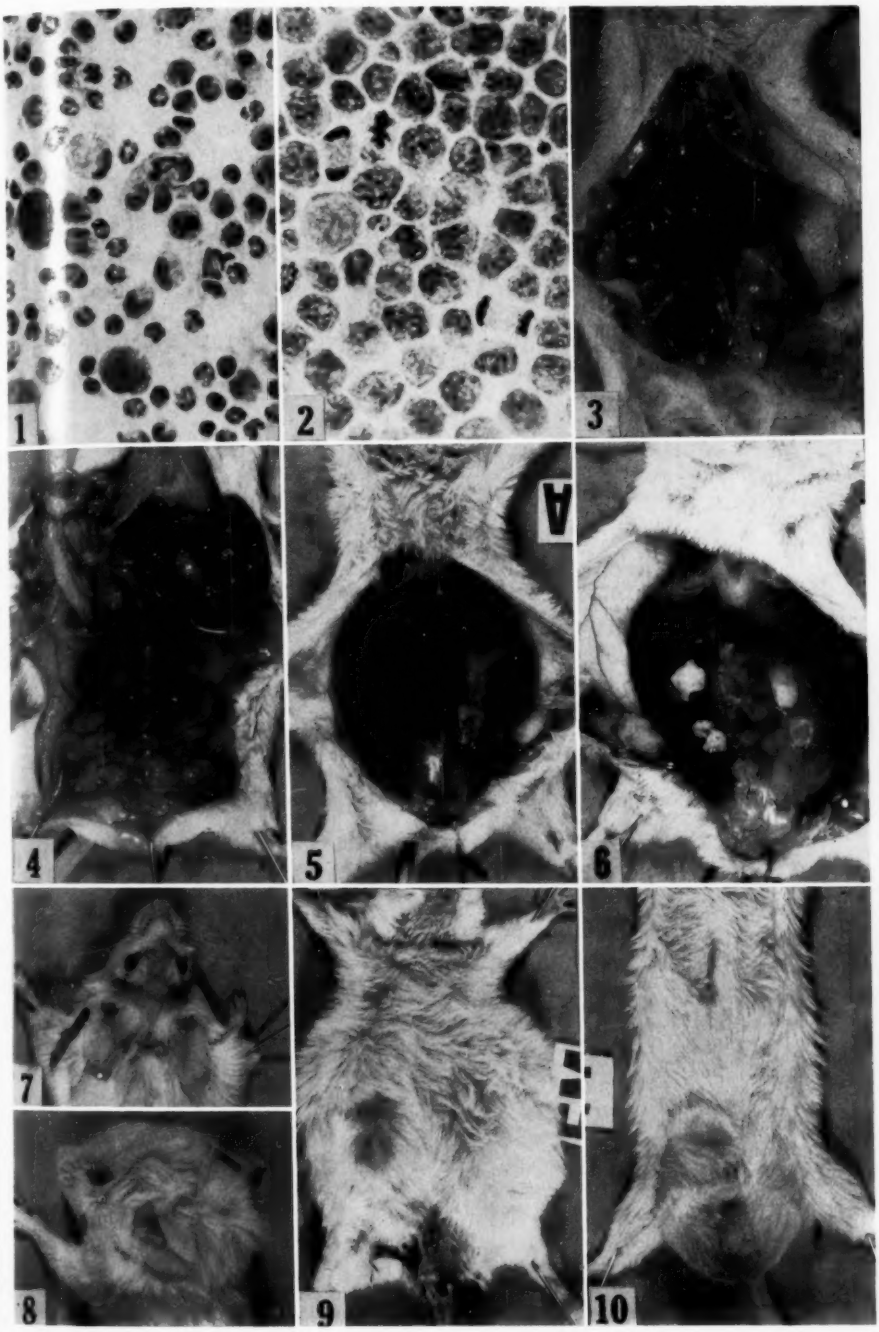
FIGURE 6.—Untreated mouse; control to figure 5.

FIGURE 7.—Scalp implant 12-day-old, treated with 4 daily peritumoral injections of 2 mg. acriflavine, starting 72 hours after inoculation.

FIGURE 8.—Untreated scalp implant; control to figure 7.

FIGURE 9.—Scrotum implant 14-day-old, treated with 4 daily peritumoral injections of 2 mg. acriflavine, starting 24 hours after inoculation.

FIGURE 10.—Untreated scrotum implant; control to figure 9.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UNIVERSITY OF CALIFORNIA
SAN DIEGO

A Study of Aminopeptidase Activity in the Stroma of Neoplastic Tissue, With a Comparison of Histochemical Techniques [1, 2]

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SUMMARY

A histochemical examination of aminopeptidase activity in over 120 benign and malignant tumors revealed intense peptidase activity in the stroma adjacent only to those tumors showing invasive characteristics. The method also demonstrated that certain human tumors, such as carcinoma of the stomach, colon, and breast, and a meningioma had aminopeptidase activity within the tumor cells proper. Aminopeptidase activity in the stroma adjacent to invasive tumor is evidence of proteolysis and suggests that a prominent mechanism of tumor invasion is by the proteolytic destruction of the stromal compartment. However, this does not imply that all invasive tumors show increased stromal aminopeptidase activity, for other enzyme mechanisms not demonstrable by the present technique may be responsible for stromal dissolution in certain tumor types. It is possible that stromal proteolysis may be elicited by a factor elaborated by tumor cells which activates the peptidase, or may represent activation as the result of an immune response. Histochemically demonstrable stromal proteolytic activity may be correlated with the invasive capacity of the tumor, but is not related to its grade of anaplasia or the extent of adjacent fibroplasia. The aminopeptidase activity demonstrated histochemically by using L-leucyl-β-naphthylamide has not been identified as "leucine" aminopeptidase since neither metal-ion dependence nor relative amino acid substrate specificity correlates with that defined by Smith *et al.* The new modification for the histochemical demonstration of aminopeptidase activity on fresh frozen and frozen-dried tissue sections is compared with a method utilizing an inhibitory, slow-coupling diazonium salt, and the inherent errors in interpretation when using the latter method are described.—J. Nat. Cancer Inst. 23: 857–872, 1959.

THE HISTOCHEMICAL demonstration of prominent aminopeptidase activity adjacent to invasive tumor was first described by Burstone (*1*), and subsequent validation of this finding has been reported (*2*, *3*). The

[1] Received for publication May 5, 1959.

[2] Presented in part at the Histochemical Society Annual Meeting, Atlantic City, N.J., April 12, 1959.

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[6] The authors are indebted to Dr. Montague Lane, of the National Cancer Institute, for his aid in supplying many of the experimental animal tumors used in this study, and to Dr. Leon Sokoloff for reviewing slides of the early rheumatoid nodule.

[7] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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significance of proteolytic activity at the periphery of invasive tumor has not been thoroughly investigated, though it has been claimed by some that the increased aminopeptidase activity of tumor stroma is related to nonspecific fibroblastic proliferation (*4*). This paper presents an evaluation of the possible role and mechanism of proteolytic activity in the stroma surrounding invasive tumor, as well as a modification of the original technique for the demonstration of aminopeptidase activity for use on fresh frozen tissue sections.

MATERIAL AND METHODS

Over 120 specimens of surgically removed human tumor tissue were histochemically examined for aminopeptidase activity. Sections from both intramuscularly and subcutaneously implanted rat and mouse tumors, of type Dunning lymphosarcoma R2788, rat, Mammary carcinoma 241-6, mouse, Lymphosarcoma L-2, mouse, thorotrast-induced Spindle-cell sarcoma H-38290-TTT, mouse, and Osteogenic sarcoma 112, mouse, were studied. Normal tissue from rat, mouse, guinea pig, dog, monkey, and human material was used, as well as tissue from human wounds excised at intervals after incision. Artificial induction of fibroplasia was stimulated in rats and mice by intradermal nuchal injection of turpentine and also by the injection of a 1 percent saline solution of carrageenin,[8] with specimens of tissue being removed 5 and 10 days later (*5*). The techniques utilized for comparison were those of Burstone (*6*), on frozen-dried material, and of Seligman *et al.* (*7*), on raw frozen tissue. Tissue taken for examination was either frozen immediately by placing the specimens on dry ice for use with the Adamstone-Taylor technique or placed in isopentane at approximately −150° C. for use with the freeze-dry method. Substrates used were synthesized in our laboratories and compared with those commercially available.[9] These included DL-alanyl-β-naphthylamide, L-leucyl-β-naphthylamide and DL-phenylalanyl-β-naphthylamide. Potential inhibitors and activators were used in the concentration described in the text.

Technique

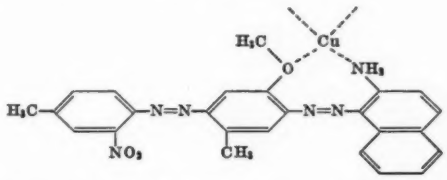
A modification of the existing techniques (*6*, *7*) for the demonstration of aminopeptidase activity was used. Of paramount importance in this reaction is the precipitation *in situ* of the released naphthylamine by a rapid-coupling diazonium salt. Such salts are characterized by Garnet GBC (diazotized *o*-aminoazotoluene) and Corinth V (diazotized 5-methyl-4-(nitro-*p*-tolylazo)-*o*-anisidine)[10] (*8*). With Corinth V, a purple-black

[8] Seakem, Type 14, furnished through the courtesy of Seaplant Chem. Corp., New Bedford, Mass.

[9] Mann Research Lab., New York, N.Y. Certain commercial aminopeptidase substrates contained free β-naphthyamine as an impurity and these gave spurious staining reactions.

[10] Corinth Fast Salt V concentrate furnished through the courtesy of General Aniline and Film Corp., New York, N.Y.

dye is deposited at the sites of aminopeptidase activity. The dye is then chelated with copper *in situ* to enhance stability of the reaction products. On chelation no bathochromic shift is noted.



TEXT-FIGURE 1.—Copper chelate of reaction product (*see 7*) of the hydrolysis of DL-alanyl-β-naphthylamide coupled with Corinth V salt.

Procedure

The incubating solution was identical to that previously described (*6*) except for the substitution of Corinth Fast Salt V (15 to 20 mg. per Coplin jar) for Garnet GBC. The substrate in most cases was DL-alanyl-β-naphthylamide [11] and in specific instances DL-phenylalanyl-β-naphthylamide was employed. Fresh frozen sections mounted on slides were used. After incubation for 10 minutes to 1 hour, the slide was placed in a 2 percent aqueous copper sulfate solution for 10 minutes, washed with water for 10 minutes, taken through 15-minute rinses in 30, 50, and 30 percent alcohol to remove decomposed diazonium salt, rinsed in water for 5 minutes, and then mounted in glycerol jelly containing several drops of 2 percent copper sulfate. When frozen-dried sections were used, the incubation time varied from 2 to 6 hours.

Specificity of the Enzyme

The relative hydrolysis rates of the substrates used for the demonstration of aminopeptidase activity have been previously estimated (*9*). These rates of hydrolysis vary markedly from those obtained by Smith who used a preparation of "leucine" aminopeptidase with comparable amino acid amides (*10*). Inhibitors for various known peptidases not previously utilized and re-evaluation of others were attempted at the following concentrations: guanidine 1.5M, Versene 0.01M, DTPA[12] 0.1M, Na salicylate 0.01M, chloromercuribenzoate 0.005M, citrate 0.005M, but no significant inhibition was noted. Attempted activation failed with the following: Co^{++} 0.001M, Mg^{++} 0.001M, Mn^{++} 0.004M, ascorbic acid 0.005M, and cysteine 0.005M. However, CN^{-} 0.01M, Pb^{++} 0.001M, Cd^{++} 0.001M, and Cu^{++} 0.001M inhibited enzymatic activity significantly. It is apparent that the enzyme demonstrated by the hydrolysis of either leucyl- or alanyl-β-naphthylamide is by definition neither "leucine" aminopeptidase (*10*) nor cathepsin III (*11*).

[11] L-leucyl-β-naphthylamide may be used also.
[12] Diethylene triaminopentaacetic acid furnished through the courtesy of Geigy Chem. Corp., Ardsley, N.Y.

Histochemical Findings

All substrates were capable of giving useful histochemical localizations. Freeze-dry sections showed sharper localizations, as well as a greater contrast, between the staining of stromal components, *i.e.*, ground substance and connective-tissue cells and fibers adjoining neoplastic and non-neoplastic tissue, as compared with fresh frozen sections. It is important to note that ground substance is better preserved in frozen-dried tissue than in fresh frozen sections. The incubation period required for the demonstration of enzymatic activity was usually shorter for fresh frozen sections than for corresponding frozen-dried material. However, when excessively prolonged incubation (2 to 4 hours) of fresh frozen tissues was carried out, *all reactive sites eventually acquired a similar staining intensity*. This did not occur when frozen-dried sections were incubated for prolonged periods (4 to 20 hours).

Normal Tissue

Fibroblasts of normal human and normal rodent skin showed slight aminopeptidase activity throughout, with most prominent activity in the immediate subepidermal zone. Intestinal villus epithelium, but not the entire villus, showed conspicuous aminopeptidase activity in the human, rodent, and dog. Intense staining of human synovial villus epithelium was noted. The alveolar-lining cells of the dog lung showed significant activity. The epithelium of the epididymis of the rat revealed strong luminal activity, while in cold formalin-fixed tissue, eosinophils in the intestinal mucosa stained deeply. In the guinea-pig kidney, vascular bundles in the medulla and only the brush border of *all* proximal tubules displayed activity. Only in the monkey, among the animals examined, was glomerular staining noted, and this staining was confined predominantly to the parietal layer of Bowman's capsule. A demonstration of aminopeptidase activity with the present method on fresh frozen rat kidney is shown (fig. 1), and compared with a serial section stained by a method previously described [(*7*), fig. 2]. Pronounced inhibition by diazonium salt and diffusion of the hydrolyzed naphthylamine as the result of slow coupling with Diazo Blue B was noted (*cf. 12*). The results obtained with the present method correspond with those previously described on frozen-dried tissues (*6*), but many vary from those reported on fresh frozen sections (*7*).

Inflammatory Tissue

An early rheumatoid nodule showed prominent aminopeptidase activity, as originally reported by Burstone (*1*). This activity was most marked in foci of "fibrinoid" necrosis and in areas of necrosis involving small vascular channels. In many sites the vessel undergoing necrosis and peripheral tissue displayed strong activity (fig. 9). Many areas of active fibroblastic proliferation in the rheumatoid nodule failed to reveal

significant aminopeptidase activity (fig. 9). In sections of healing wounds fibroblasts were minimally stained, but macrophagic and inflammatory cell staining was prominent. Sections were taken from sites of the healing incision at intervals corresponding to the times of maximal fibroblastic proliferation (*13*), and no prominent enzymatic activity of fibroblasts was noted. A comparison with older, less plump fibroblasts in the normal corium showed enzymatic activity of a similar degree. In an area of active cicatrix formation, fibroblasts were essentially unreactive (fig. 3). Granulation tissue at the bases of an acute gastric ulcer, a decubitus ulcer, and a dental granuloma contained the greatest activity in inflammatory cells and in small proliferating vascular channels, as previously reported (*1*). In artificially induced fibroplasia stimulated by carrageenin (*5*) marked fibroplasia was noted, but again aminopeptidase activity was minimal and most areas of proliferating fibroblasts showed insignificant staining, though the enzymatic activity in macrophages was often intense. A similar observation was made in rats treated with intradermal injections of turpentine.

Neoplasms

Specimens of benign and malignant tumors as well as inflammatory and retention cysts from human surgical material were examined (table 1). The localization of aminopeptidase activity was, in many instances, compared with the hematoxylin- and eosin-stained consecutive section or sections stained by the Feulgen reaction.

Adenocarcinoma.—Intense stromal activity in a specimen of rectal carcinoma was noted (fig. 4), while no staining in the luminal borders of tumor acini was evident. In colonic carcinoma, however, the staining of the luminal borders of neoplastic glands (fig. 10) was seen in all cases examined, while in certain instances intense stromal activity was also present either in areas of invasion of the submucosa or in stromal tissue isolated by the neoplasm. It was possible in several cases of early malignant degeneration of a rectal polyp to locate precisely the area of invasion by the stromal staining visible when the tissue section was viewed *grossly*. The greatest intensity of staining occurred nearest the invasive tumor cells, and was less intense at a distance from the area of invasion. The adenocarcinoma of the stomach showed conspicuous activity of tumor cells with minimal activity of invaded stroma. In the follicular and papillary carcinomas of the thyroid, evidence of stromal activity was present only in areas of *active* invasion by neoplastic microfollicles or individual malignant cells.

Epidermoid carcinomas.—Pronounced stromal activity was apparent in all cases of laryngeal carcinoma (fig. 5), epidermoid carcinoma of the cervix, and in squamous carcinoma of the skin (fig. 6), while the sections of lingual carcinoma revealed intense activity where the tumor invaded striated muscle. In many sites where there was marked fibroblastic activity surrounding sheets and groups of malignant cells, no peptidase

TABLE 1.—Aminopeptidase activity in neoplasias

Neoplasms	Number cases	Stromal activity	Tumor cell or epithelial activity
Malignant			
Adenocarcinoma—rectum	3	4+	0
Adenocarcinoma—lung	1	2+	0
Adenocarcinoma—colon	7	0–4+	4+
Adenocarcinoma—stomach	1	1+	4+
Adenocarcinoma—uterus	2	2+	0
Adenocarcinoma—salivary gland	1	4+	0
Follicular and papillary carcinoma—thyroid	6	0–4+	0
Epidermoid carcinoma—pharynx	2	4+	0
Epidermoid carcinoma—anus	1	3+	0
Squamous carcinoma—vulva	1	4+	0
Squamous carcinoma—lip	1	3+	0
Epidermoid carcinoma—epiglottis	1	2+	3+
Epidermoid carcinoma—tongue and mouth	12	4+	0
Epidermoid carcinoma—cervix	17	4+	0
Epidermoid carcinoma—trachea	1	4+	0
Epidermoid carcinoma—larynx	9	2+–4+	0
Epidermoid carcinoma—antrum and paranasal sinuses	3	4+	0
Epidermoid carcinoma—tonsil	2	4+	0
Squamous carcinoma—skin	8	2+–4+	0
Basal-cell carcinoma—skin	5	3+–4+	1+
Melanocarcinoma	6	4+	0–2+
Lobular carcinoma—breast	1	3+	0
Scirrhous carcinoma—breast	1	0	2+
Hepatocellular carcinoma	2	2+–4+	0
Adrenal cortical carcinoma	1	0	0
Lymphosarcoma	1	1+	0
Benign and hyperplasia			
Desmoid—abdominal wall	2	0	0
Gingival polyp	1	0	0
Lipoma	1	0	0
Islet-cell adenoma—pancreas	2	0	0
Parathyroid adenoma	2	0	0
Hemangioma—capillary	1	0	0
Ganglioneuroma	1	0	0
Leiomyoma—uterus	2	0	0
Fibroma—extremity	2	0	0
Mixed tumor—parotid	5	0	0
Polyp—colon	1	0	0
Meningioma	1	0	4+
Fibrocystic disease—breast	2	0	4+
Prostatic hyperplasia	2	0	4+

activity was demonstrable. In most cases peptidase activity was not present in all stromal areas surrounding the tumor, occasionally being only focal in character (fig. 11). Leukocytic infiltration was observed in only a small percentage of cases. In some areas foci of necrosis within the tumor contained moderate aminopeptidase activity.

Other malignancies.—Examination of mixed tumors of the parotid gland revealed no stromal activity, but in an adenocarcinoma of the salivary gland (where marked invasion occurred) strong peptidase activity was observed. In a meningiothelial meningioma, prominent activity was confined entirely to the tumor cells (fig. 12). Examination of sections of melanocarcinoma revealed strong activity adjacent to

advancing clusters of malignant nevus cells (fig. 7), while in several instances moderate staining of the melanoma cells also was noted.

Benign tumors and hyperplasia.—The parathyroid adenomas showed no peptidase activity. This lack of activity was also evident in the pancreatic islet-cell adenomas with clinical hypoglycemia. In the fibrocystic disease of the breast, with areas of sclerosing adenosis and papillomatosis, prominent activity was limited to the duct and lobular epithelium. Also, in prostatic hyperplasia (benign prostatic hypertrophy) the epithelium and luminal contents of the glands stained, while the periglandular tissue activity was minimal. Desmoid tumors of the abdominal wall and leiomyomas of the uterus were unreactive in areas of greatest fibroblastic and smooth muscle proliferation.

Experimental tumors.—In the intramuscularly transplanted, thorotrast-induced, spindle-cell sarcoma of mice, intense peptidase activity was present at the boundary of invasive tumor while tumor cells remained unreactive. The osteoclasts in the osteogenic sarcoma of mice, transplanted intramuscularly, contained the enzyme, while the remaining tumor cells were unreactive, but at the sites of invasion of stroma at the tumor periphery peptidase activity was present. Stromal activity was also present in intramuscularly transplanted mammary carcinomas at the sites of stromal and striated muscle invasion (fig. 8). Moderate peptidase activity was observed at the invading border of both types of lymphosarcomas; however, most prominent was a strong staining reaction surrounding individual tumor cells in numerous areas within the tumor.

Other conditions.—Epithelial retention cysts and radicular cysts revealed aminopeptidase activity only in scattered inflammatory cells and vascular sprouts in the surrounding tissue.

DISCUSSION

From the findings of inhibitor and activator studies, it is evident that the enzyme demonstrated by the use of L-leucyl-β-naphthylamide as substrate is not identifiable with "leucine" aminopeptidase as defined by Smith *et al.* (*10*, *14*), *i.e.*, as a metal-dependent enzyme inhibited by Versene and citrate. The claim by Nachlas, Crawford, and Seligman (*7*) that an enzyme hydrolyzing L-leucyl-β-naphthylamide is *ipso facto* "leucine" aminopeptidase is not consistent with the observed facts. Other peptidases are known to split leucyl peptides and these have not been identifiable with "leucine" aminopeptidase (*15*). The finding that DL-alanyl-β-naphthylamide is hydrolyzed more rapidly than L-leucyl-β-naphthylamide in the histochemical preparation (*9*) is contrary to the described substrate specificity of "leucine" aminopeptidase (*10*). The histochemically demonstrable aminopeptidase may be more closely associated with one described by Greenstein *et al.* (*16*), which hydrolyzes amino acid-4-biphenylene amides. Recent studies by Waldschmidt-Leitz and Keller (*17*) have shown that so-called "leucine" aminopeptidase

is actually a mixture of peptidases, so it would indeed be remarkable if any histochemical substrate would exhibit preference for this heterogeneous system. Therefore, if "leucine" aminopeptidase does not exist as a specific enzyme [and evidence to the contrary is indicated (*17a*)], then the specific terminology should not be used either biochemically (*18*) or histochemically. If "leucine" aminopeptidase should be found to exist as a specific enzyme, then the histochemically demonstrable enzyme system using amino acid-β-naphthylamides must be shown to have the same substrate specificity and evidence of metal dependence as that which defines the enzyme. Otherwise identity is not possible. It is of interest to note that all three substrates gave essentially identical distribution of activity in the tissue examined, and it would appear, therefore, that the leucyl residue does not confer specificity to the histochemical reaction.

The need for a rapid coupling, noninhibitory diazonium salt, such as Corinth V or Garnet GBC (*8*), for the histochemical demonstration of enzymatic activity is apparent when comparison of localization with the results obtained with the inhibitory, slow-coupling diazonium salt [Diazo Blue B (*8*)] on comparably treated tissue is made. The sensitivity and accuracy of the present method becomes most evident with the finding of localization of aminopeptidase activity in the medullary vascular bundles of raw frozen guinea-pig kidney and limitation of activity to the luminal border of *all* proximal tubules of raw frozen rat kidney. These findings were not previously noted in an investigation in which *comparably treated* tissues were used (*7*). Coupling with a slow reacting diazonium salt will produce diffusion artifacts and false-positive localization of enzymatic activity (*12*). Thus, spurious staining of stroma adjacent to sites of high activity, *e.g.*, the breast and the prostatic duct epithelium, may result.

The finding of increased activity of raw frozen sections of tissue as compared with frozen-dried material signifies either an activation of aminopeptidase by freezing and thawing or an inhibition of activity during the freeze-dry procedure or subsequent embedding. Which of these two factors accounts for the increased incubation time necessary for the demonstration of enzymatic activity in freeze-dry material is not known. But the identical localization of aminopeptidase activity, by the present method, on both frozen-dried and fresh frozen sections is indicative of the accuracy of localization described on frozen-dried material alone (*1*, *6*).

With the localization of aminopeptidase activity to known sites of breakdown of stroma, especially that adjacent to invading tumor (*19*), it becomes evident that this enzyme is involved in proteolysis rather than synthesis. As Sylvén states: ". . . microscopical changes usually appear to begin at a distance of one or a few millimeters from the tumor periphery and become more marked close to the tumor border. The changes include *interstitial edema, swelling and dispersal of preformed collagen, and muscle fiber bundles*, and various *cytological changes of fibro-*

blasts, ranging from regeneration to cytolysis" (*20*, p. 17). The *in vitro* preponderance of proteolysis to synthesis in *exopeptidase* reactions is a biochemical fact (*21*, p. 714). As evidenced by our findings in areas of necrosis in rheumatoid nodules, as well as in the findings in sites of actively proliferating dermatoses and dermatoses associated with breakdown of ground substance (*22*, *23*), aminopeptidase activity in these foci is also indicative of proteolysis. This interpretation is of extreme importance in evaluating the mechanism of spread of tumor cells, for proteolytic activity preceding tumor cells would destroy one protective mechanism against tumor spread—the integrity of the stromal compartment. As Berenblum states: "Even as a mere contributory factor, rapidity of growth only affords the *scope* for invasion by providing new tissue. The *mechanism* [for tumor invasion] involves other factors." (*24*, p. 454). "The only possible mechanism left [for the invasive ability of malignant tumors] is that the malignant tumor cell elaborates some extracellular lytic products, capable of dissolving or partially destroying the tissue being invaded." (*24*, p. 456). Aminopeptidase activity is probably only *one* of a series of proteolytic enzymatic reactions present in the stroma adjacent to invasive tumor and such stromal breakdown, therefore, may be the result of a continuum of enzymatic degradative processes. Sylvén and Malmgren (*20*), in a quantitative study of hundreds of experimental tumors, have noted this relationship of tumor invasiveness and the activity of various proteolytic enzymes.

The mechanism for the establishment of proteolytic activity adjacent to tumor need not necessarily be found in the elaboration of a lytic substance *per se* by the tumor cells. In only certain tumor types, as we and others (*3*, *25*) have shown, is aminopeptidase activity localized in the tumor cell itself. *It is more likely that the prominent increase in stromal proteolytic activity is the result of an induction or activation of proteolytic enzymes by a factor or factors elaborated by tumor cells.* It would therefore appear that the histochemically demonstrable stromal aminopeptidase may be activated by a factor elaborated by tumor cells, in the same manner as fibrinolysin is indirectly activated by streptokinase (*26*, p. 25). The possibility that the cells of invasive tumors elaborate a cofactor or coenzyme required for the activity of the histochemically demonstrable stromal peptidase must not be eliminated. Although we have been unable to identify the histochemically demonstrable peptidase with an enzyme known to be activated by complement (*27*), it is possible that a local tissue immune reaction may be stimulating aminopeptidase activity and consequent connective tissue and stromal dissolution. Green (*28*) has expressed this possibility in stating, ". . . the spread of tumors might be in part due to active aggression and cell infiltrations around them, expressions of an immune response." It is evident from the complete lack of correlation between the degree and extent of fibroplasia and aminopeptidase activity that aminopeptidase activity in the stroma adjacent to tumors is not "characteristic of fibroblastic activity anywhere" (*4*, *29*). From our findings it is apparent that many areas of

stroma surrounding invasive tumor sites show marked fibroplasia, but no evident aminopeptidase activity. Contrariwise, in areas of necrosis, *e.g.*, in the rheumatoid nodule, marked aminopeptidase activity is present in many sites lacking fibroplasia. In experimentally induced granulomas, examined at various stages of their development, insignificant aminopeptidase activity within fibroblasts has been reported (*30*). It follows, therefore, that a relationship between the degree and extent of aminopeptidase activity and nonspecific fibroblastic proliferation cannot be made. The heterogeneous distribution of aminopeptidase activity, as seen in a number of invasive tumors in the present study, is consistent with the known pathological fact that, when stromal destruction adjacent to tumor tissue occurs, it is not everywhere uniform. In some areas adjoining tumor cells, no histological evidence of stromal destruction is present.

From our findings and those of others (*2*, *22*, *23*, *25*) it is unlikely that increased aminopeptidase activity in stroma adjacent to tumor is indicative of the grade of anaplasia (malignancy) of the tumor, for certain locally invasive, benign neoplasms have been demonstrated to have prominent stromal activity (*22*). There is, however, every indication that a correlation between stromal aminopeptidase activity and the local invasiveness of tumor cells exists (*2*). *This does not signify or connote in any way that all rapidly invasive tumors show increased stromal aminopeptidase activity, for in specific tumor types other enzyme mechanisms* (*31*) *may be responsible for stromal dissolution, i.e., dissolution of ground substance and/or connective-tissue cells and fibers.*

REFERENCES

(*1*) BURSTONE, M. S.: Histochemical demonstration of proteolytic activity in human neoplasms. J. Nat. Cancer Inst. 16: 1149–1161, 1956.

(*2*) BRAUN-FALCO, O.: Histochemical demonstration of aminopeptidase in normal and pathological skin. (Letters to the editor.) J. Histochem. 5: 94, 1957.

(*3*) WILLIGHAGEN, R. G. J., and PLANTEYDT, H. T.: Aminopeptidase activity in cancer cells. Nature, London 183: 263–264, 1959.

(*4*) MONIS, B., NACHLAS, M. M., and SELIGMAN, A. M.: Histochemical observations on leucine aminopeptidase in diseased tissues. (Abstract.) J. Histochem. 6: 395, 1958.

(*5*) WILLIAMS, G.: A histological study of the connective-tissue reaction to carrageenin. J. Path. & Bact. 73: 557–563, 1957.

(*6*) BURSTONE, M. S., and FOLK, J. E.: Histochemical demonstration of aminopeptidase. J. Histochem. 4: 217–226, 1956.

(*7*) NACHLAS, M. M., CRAWFORD, D. T., and SELIGMAN, A. M.: The histochemical demonstration of leucine aminopeptidase. J. Histochem. 5: 264–278, 1957.

(*8*) The Dyeing of Cotton with Brenthol Dyestuffs. London, Imperial Chemical Industries, Ltd., Dyestuffs Division, 1951.

(*9*) BURSTONE, M. S.: Histochemical comparison of chromogenic substrates for aminopeptidase. (Abstract.) J. Histochem. 4: 421–433, 1956.

(*10*) SMITH, E. L.: Aminopeptidase. *In* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.). New York, Academic Press Inc., 1955, vol. 2, Art. 9.

(11) FRUTON, J. S., IRVING, G. W., JR., and BERGMANN, M.: On the proteolytic enzymes of animal tissues. II. The composite nature of beef spleen cathepsin. J. Biol. Chem. 138: 249–262, 1941.

(12) BURSTONE, M. S.: The relationship between fixation and techniques for the histochemical localization of hydrolytic enzymes. J. Histochem. 6: 322–339, 1958.

(13) AREY, L. B.: Wound healing. Physiol. Rev. 16: 327–406, 1936.

(14) SMITH, E. L., and SPACKMAN, D. H.: Leucine aminopeptidase. V. Activation, specificity and mechanism of action. J. Biol. Chem. 212: 271–299, 1955.

(15) FLEISHER, G. A.: Peptidase in human blood. II. The hydrolysis of additional peptides by normal serum. J. Biol. Chem. 206: 637–640, 1954,

(16) GREENSTEIN, J. P., WINITZ, M., and BIRNBAUM, S. M.: Studies on carcinogenic amines. I. L- and D- amino acid derivatives of 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl, and their susceptibility to hydrolysis by tissue homogenates. J. Am. Chem. Soc. 77: 5721–5725, 1955.

(17) WALDSCHMIDT-LEITZ, E., and KELLER, L.: Zur Frage der Existenz einer Leucylpeptidase. Ztschr. physiol. Chem., Hoppe-Seyler's 309: 228–238, 1957.

(17a) VESCIA, A.: The specificity of aminopeptidase. *In* Résumés communs. 3e Congress Internat. Biochim. Brussels, 1955, p. 37.

(18) GREENSTEIN, J. P., and BIRNBAUM, S. M.: Personal communications.

(19) PAPILIAN, V. V., and GRÜN, I.: Die Veränderungen der argyrophilen Grundsubstanz bei malignen Prozessen. Ztschr. Ges. Inn. Med. 13: 626–628, 1958.

(20) SYLVÉN, B., and MALMGREN, H.: The histological distribution of proteinase and peptidase activity in solid tumor transplants. Acta radiol. Supp. 154: 1–224, 1957.

(21) FRUTON, J. S., and SIMMONDS, S.: General Biochemistry, 2nd ed. New York, J. Wiley & Sons, 1953, 952 pp.

(22) BRAUN-FALCO, O.: Histochemische Aminopeptidase-Darstellung in normaler Haut, bei Psoriasis, Dermatitis, Basaliom, spinozellulärem Karzinom and Molluscum sebaceum. Dermat. Wchnschr. 134: 1341–1349, 1956.

(23) ———: Über die Histotopographie der Aminopeptidase bei Hauttumoren. Klin. Wchnschr. 35: 50–51, 1957.

(24) BERENBLUM, I.: The nature of tumor growth. *In* General Pathology, 2nd ed. (Florey, H., ed.). London, Lloyd-Lake Ltd., 1958, chapt. 21.

(25) WATTENBERG, L. W.: Histochemical study of aminopeptidase in metaplasia and carcinoma of the stomach. A.M.A. Arch. Path. 67: 281–286, 1959.

(26) MÜLLERTZ, S.: Mechanism of activation and effect of plasmin in blood. Acta physiol. scandinav. 130: 13–65, 1956.

(27) BECKER, E. L.: Enzymatic mechanisms in the action of complement. J. Allergy 29: 191–200, 1958.

(28) GREEN, H. N.: Immunological basis of carcinogenesis. Brit. M. Bull. 14: 101–105, 1958.

(29) MONIS, B., NACHLAS, M. M., and SELIGMAN, A. M.: Study of leucine aminopeptidase in neoplastic and inflammatory tissues with a new histochemical method. Cancer 12: 601–608, 1959.

(30) GEDIGK, P., and BONTKE, E.: Über die Enzymaktivität im Fremdkörpergranulationgewebe. Virchows Arch. 330: 538–568, 1957.

(31) MCCUTCHEON, M., and COMAN, D. R.: Spreading factor in human carcinomas. Cancer Res. 7: 379–382, 1947.

PLATE 70

FIGURE 1.—Rat kidney showing staining of luminal border of *all* proximal tubules. Fresh frozen; 1-hour incubation; Corinth V, leucyl substrate.

FIGURE 2.—Consecutive section to figure 1. *Note* inhibition of reaction in outer cortex and diffuse staining of tubules in inner zone. Fresh frozen; 2-hour incubation; Diazo Blue B, leucyl substrate (7).

FIGURE 3.—Extensive fibroblastic proliferation in human wound healing. *Note* peptidase activity in fibroblasts in subepidermal zone and relative inactivity in areas of marked dermal fibroplasia. Fresh frozen; 1-hour incubation; Corinth V, leucyl substrate.

FIGURE 4.—Prominent peptidase activity only in stroma of rectal carcinoma. Human. Frozen-dried; 3-hour incubation; Garnet GBC, alanyl substrate.

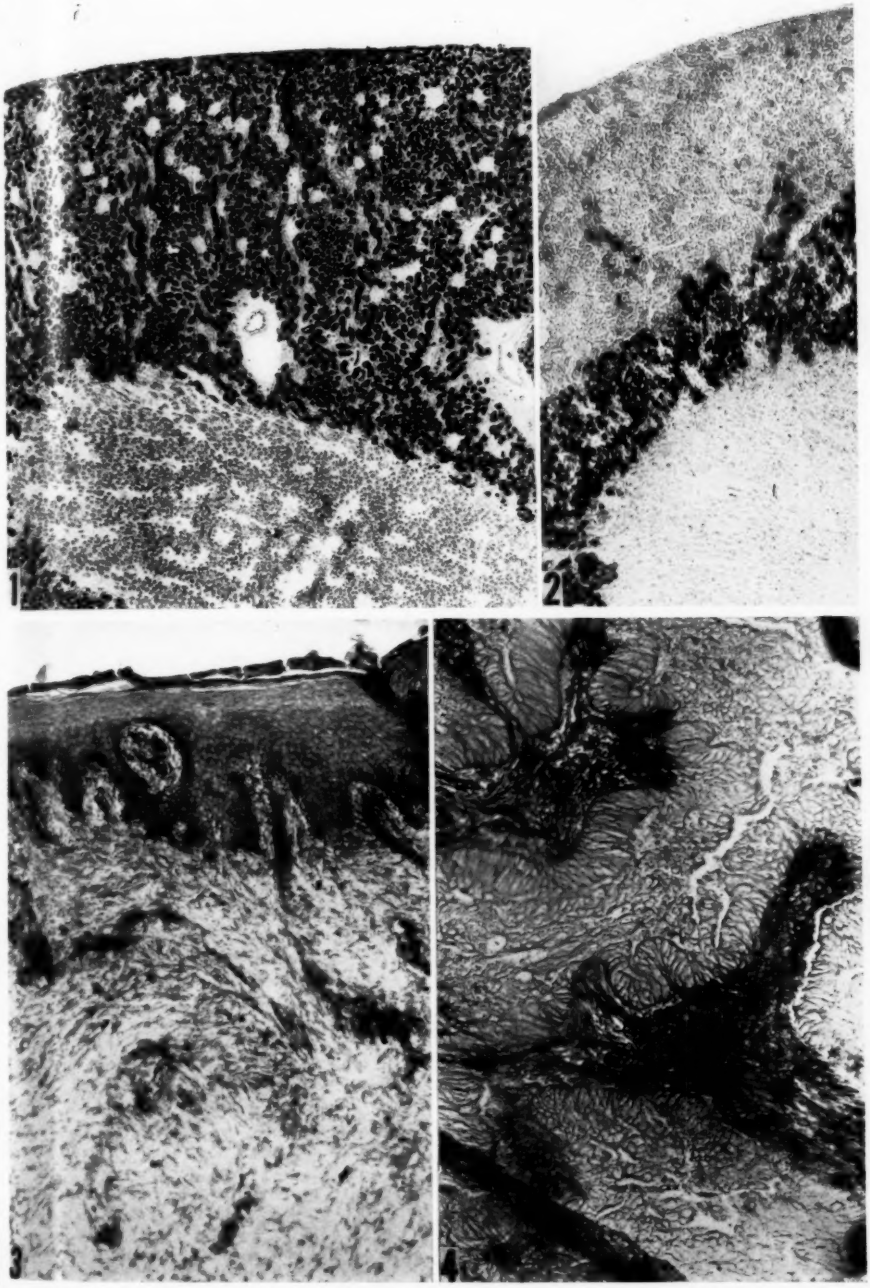


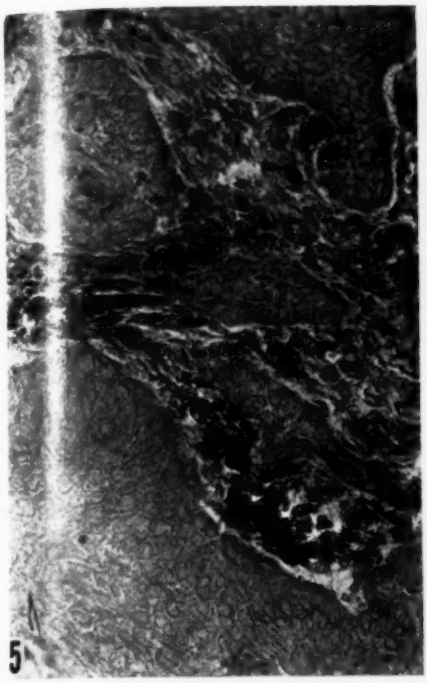
PLATE 71

FIGURE 5.—Aminopeptidase activity immediately adjacent to invasive epidermoid carcinoma of larynx. Neighboring areas of fibroblastic proliferation are inactive (*top*). Human. Frozen-dried; 3-hour incubation; Garnet GBC, alanyl substrate.

FIGURE 6.—Aminopeptidase activity in stroma infiltrated by squamous carcinoma of skin. Human. Fresh frozen; 45-minute incubation; Corinth V, alanyl substrate.

FIGURE 7.—Aminopeptidase activity in stroma infiltrated by melanocarcinoma. Human. Frozen-dried; 4-hour incubation; Garnet GBC, alanyl substrate.

FIGURE 8.—Aminopeptidase activity in stroma at site of infiltration of muscle (*top*) by mammary carcinoma (*bottom*). Mouse. Fresh frozen; 1-hour incubation; Corinth V, alanyl substrate.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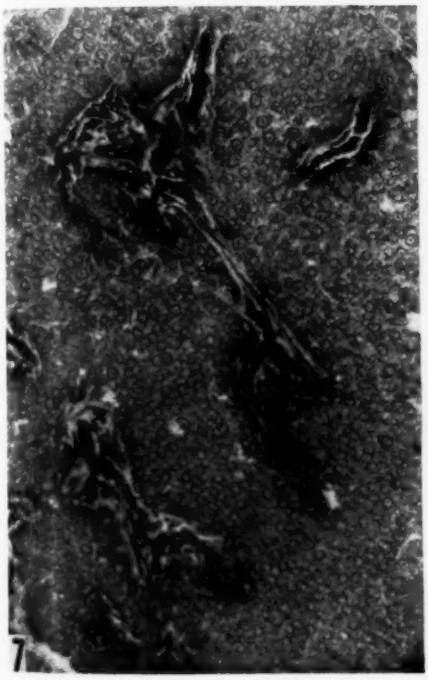
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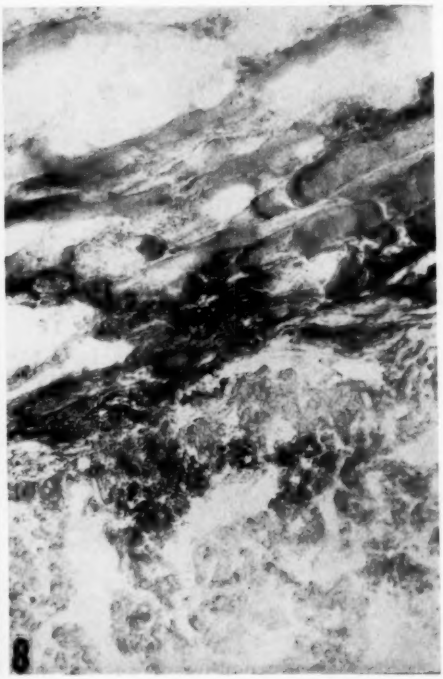
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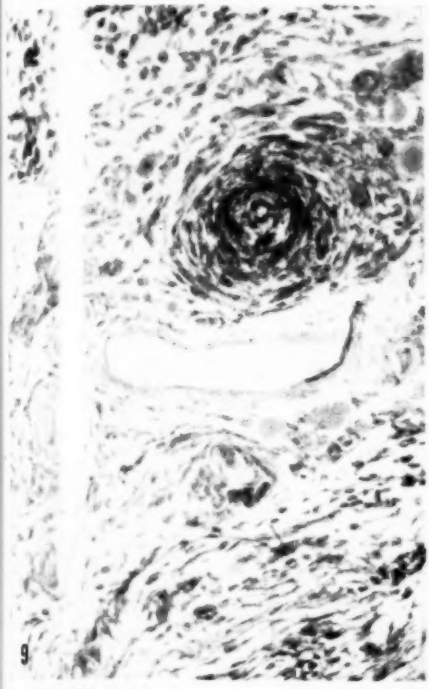
PLATE 72

FIGURE 9.—Aminopeptidase activity in wall of small vascular channel surrounded by "fibrinoid" necrosis in early rheumatoid nodule. *Note* foci of fibroblastic proliferation without enzymatic activity (*bottom*). Human. Fresh frozen; 30-minute incubation; Corinth V, alanyl substrate.

FIGURE 10.—Aminopeptidase activity localized to the luminal borders of a well-differentiated adenocarcinoma of sigmoid colon. *Note* unstained fibroblasts in surrounding stroma. Human. Frozen-dried; 3-hour incubation; Garnet GBC, alanyl substrate.

FIGURE 11.—Aminopeptidase activity localized to focal stroma area without fibroblastic activity adjacent to an epidermoid carcinoma of cervix. Neighboring areas of stroma show no peptidase activity. Human. Fresh frozen; 1-hour incubation; Corinth V, alanyl substrate.

FIGURE 12.—Aminopeptidase activity localized exclusively to tumor cells of a meningioma. Human. Frozen-dried; 4-hour incubation; Garnet GBC, alanyl substrate.



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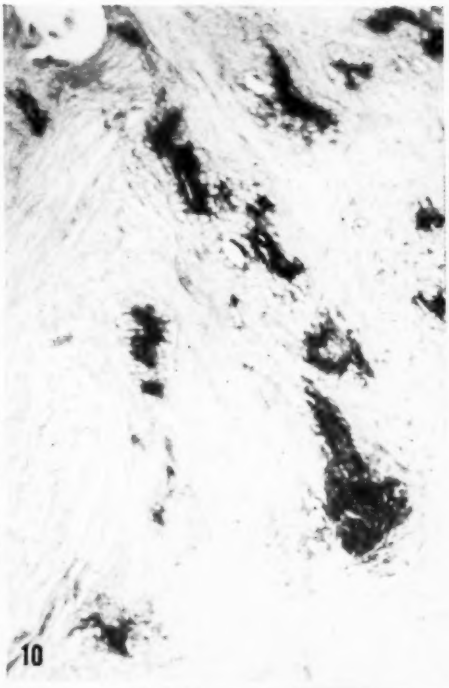

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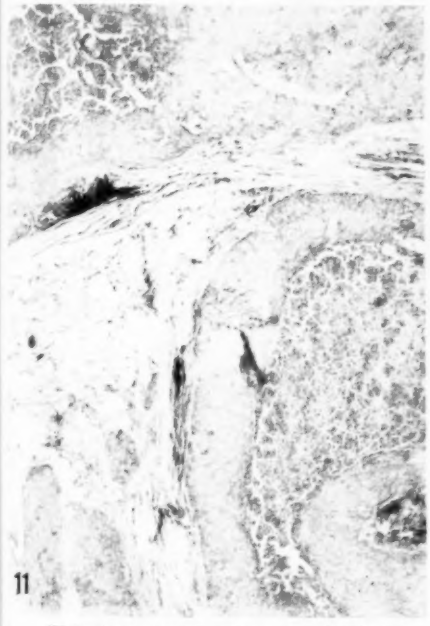

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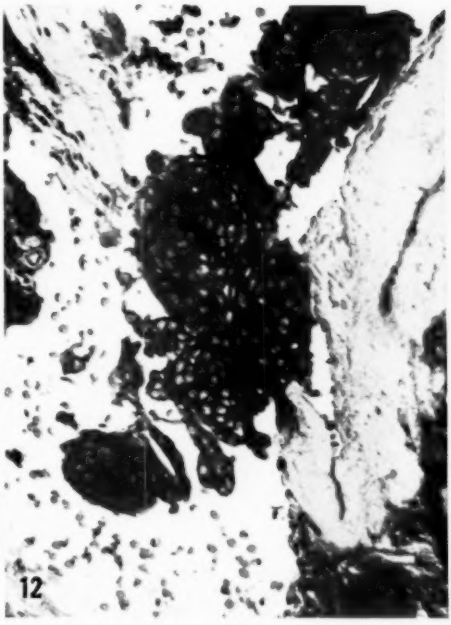

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GLENNER, BURSTONE, AND MEYER